Genetic and phenotypic characterisation of mumps virus

Thesis

UNRESTRICTED

GENETIC AND PHENOTYPIC CHARACTERISATION OF MUMPS VIRUS

BY

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ABSTRACT

Virological techniques have previously been used to investigate mumps virus (MuV) replication *in vitro* and have led to a rapid expansion of our understanding of this virus. The cell line of choice has been Vero cells, however, recent studies have concluded that variants of the original MuV are selected upon passage in Vero cells. The aim of this thesis is to describe the effect of cell substrate on MuVs through genotypic and phenotypic analysis after growth of MuVs in different host cells.

Data is presented which demonstrates that MuVs passaged in B95a or HeLa cells are of greater fitness than the parental Vero cell-grown viruses, when assayed to determine the titre of infectious virus produced. No adaptation period was required for growth of MuVs in B95a cells with the exception of one variant of the JL vaccine. An adaptation period was required for growth in HeLa cells. Only one of the viruses studied, a variant derived from Urabe vaccine, grew to a high infectivity titre in MRC-5 cells. Growth occurred after an initial adaptation period, suggesting a narrow bottleneck for virus growth in these cells and amino acid 431 in the HN protein is implicated in the adaptation to these cells. Sequence analysis of the two envelope glycoproteins implicates amino acids 92, 205, 255, 347, 392 and 526 in the HN protein of B95a and HeLa derived viruses as being responsible for phenotypic changes in plaque morphology and antigenicity but do not solely account for host cell tropism.

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ABBREVIATIONS

A (nucleoside)	Adenosine
APS	Ammonium persulphate
BPB	bromophenol blue
bPIV	Bovine parainfluenza virus
bRSV	Bovine respiratory syncytial virus
CNS	Central nervous system
C (nucleoside)	Cytosine
CDCP	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CDV	Canine distemper virus
CEF	Chick embryo fibroblast
CMC	Carboxy-methylcellulose
CMI	Cell mediated immunity
CO_2	Carbon dioxide
CPE	Cytopathic effect
CSF	Cerebrospinal fluid
dATP	Deoxyadenosine triphospate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphophate
dTTP	Deoxythymidine triphosphate
ddATP	Dideoxyadenosine triphospate
ddCTP	Dideoxycytosine triphosphate
ddGTP	Dideoxyguanosine triphophate
ddTTP	Dideoxythymidine triphosphate
DI	Defective interfering
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetra-acetic acid
EIA	Enzyme immunoassays
ER	Endoplasmic reticulum
F	Fusion
FCS	Foetal calf serum
G (nucleoside)	Guanosine
GDW	Glass distilled water
H_2O	Water
HIV	Human immunodeficiency virus
HA	Haemagglutinin
HN	Haemagglutinin-neuraminidase
HPIV	Human parainfluenza virus
HR	Heptad repeat
hRSV	Human respiratory syncytial virus
IgA	Immunoglobin A
IgG	Immunoglobin G
IgM	Immunoglobin M
IMS	Industrial methylated spirit
JL-2	Jeryl Lynn-2
JL-5	Jeryl Lynn-5
L	Large

M	Matrix
MAb	Monoclonal antibody
MAR	Monoclonal antibody resistant
MEM	Minimum essential medium
MDCK	Madin Darby canine kidney
MNVT	Monkey neurovirulence test
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSD	Merck, Sharpe and Dohme
MuV	Mumps virus
MV	Measles virus
NA	Neuraminidase
NDV	Newcastle disease virus
NEP	Neutralisation end point
NP	Nucleoprotein
ORF	Open reading frame
P	Phosphoprotein
PBS'A'	Phosphate buffered saline 'A'
PHLS	Public Health Laboratory Service
PNK	Polynucleotide Kinase
RBC	Red blood cell
RBS	Receptor binding site
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPV	Rinderpest virus
SDS	Sodium dodecyl sulphate
SeV	Sendai virus
SH	Small hydrophobic
SSPE	Subacute sclerosing panencephalitis
SV5	Simian virus 5
T (nucleoside)	Thymidine
TBE	Tris, Boric acid, EDTA
U (nucleoside)	Uridine
Ur PT1	Urabe plaque type 1
Ur PT3	Urabe plaque type 3
V	V protein
VSV	Vesicular stomatitis virus

CHAPTER I

INTRODUCTION

1.1 GENERAL HISTORY OF MUMPS

Mumps, as an illness, has been recognised since the 5th century BC when Hippocrates described epidemics of diseases with symptoms characteristic of mumps, as we now know it. A characteristic symptom of mumps is parotitis, or swelling of the parotid gland. In 1790, a physician, Robert Hamilton, described an illness, "called mumps by the common people of England", which he called *angina maxillaris*. Hamilton was the first to document the symptoms of the disease and to associate the involvement of the central nervous system (CNS) (Hamilton., 1790). The agent responsible for this disease was not identified until 1935 when Johnson and Goodpasture demonstrated that mumps was the result of a viral infection (Johnson *et al.*, 1935). Saliva, collected from a human with parotitis, was inoculated into the parotid glands of *Macacus rhesus* monkeys by way of the Stensons' duct. The animals were sacrificed after bilateral parotid swellings were observed and a homogenate of the infected glands produced. This emulsion was used to inoculate animals for further passage. After eleven passages in the monkey, an emulsion of parotid glands, collected from the infected monkeys, was inoculated into humans by way of the oral cavity. Six of the human patients manifested typical clinical mumps. Johnson and Goodpasture concluded that mumps was due to a filterable virus present in saliva, at least in early stages of the disease, which was capable of inducing parotitis in *M. rhesus* monkeys when inoculated directly into the Stenson's ducts. The authors also concluded that mumps could be contracted by contact with the virus through saliva.

Isolation of the virus causing mumps led to an understanding of the physical and molecular properties of the virus, its epidemiology and pathogenesis and to the production of a vaccine, which was introduced in the U.S.A. in 1967 and then in the rest of the world leading to a steady decline in the cases of mumps where the vaccine is used appropriately.

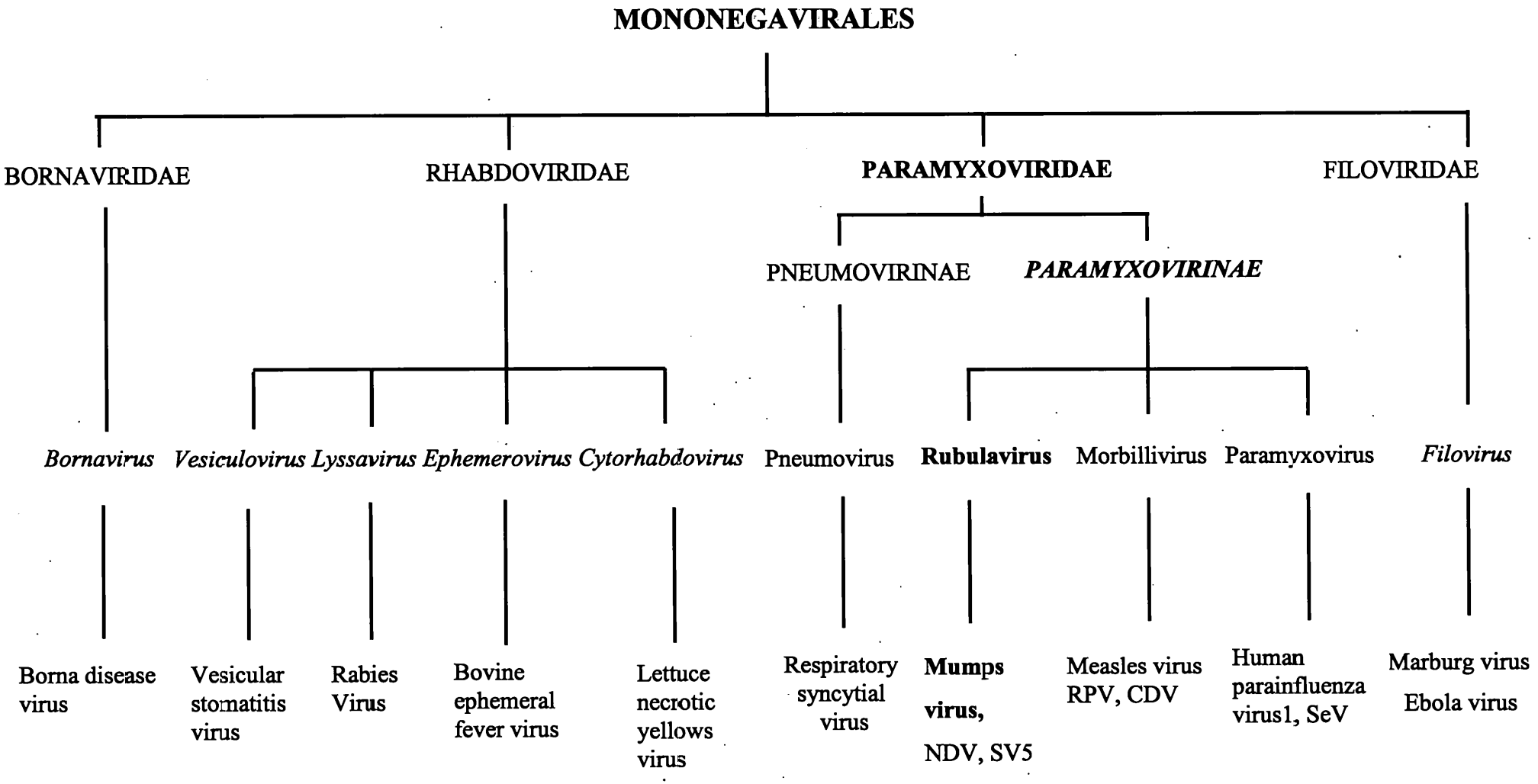
1.2 THE MUMPS VIRUS

1.2.1 CLASSIFICATION

The negative sense, single-strand RNA genome of mumps virus (MuV) is characteristic of viruses within the virus super-family *Mononegavirales*, which comprises four families; *Rhabdoviridae*, *Paramyxoviridae*, *Bornaviridae* and *Filoviridae* (Pringle and Easton, 1997). Originally, MuV was classified alongside influenza virus in a *Myxoviridae* family, due to the ability of both viruses to cause haemagglutination and other similar characteristics. However, MuV is currently classified in the *Rubulavirus* genus (Figure 1.1), within the *Paramyxovirinae* subfamily of the *Paramyxoviridae* family of viruses (Rima *et al.*, 1995). The name *paramxyo* comes from the Greek, *para* meaning close to (referring to the viruses relationship with other myxoviruses) and *myxa* meaning mucous (referring to the site of infection of the virus). The *Paramyxoviridae* family consists of two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. The *Paramyxovirinae* contains three genera, *Paramyxovirus*, *Rubulavirus* and *Morbillivirus*. The *Pneumovirinae* subfamily contains only one genus, the *Pneumovirus*. This classification system is based on several features including genome organisation, morphological characteristics, activities of the expressed proteins and sequence relationships of the encoded proteins.

The rubulavirus genus contains Newcastle disease virus (NDV) which is quite distinct, and it has recently been suggested through sequence analysis that it be placed in a separate genus (de Leeuw *et al.*, 1999). Other reasons given were that NDV edits its phosphoprotein (P) gene mRNA from P to a V protein, as do measles virus (MV) and Sendai Virus (SeV), rather than from the V protein mRNA to the P, as does MuV and other rubulaviruses (Steward *et al.*, 1993). Secondly, paramyxovirus genomes are replicated only when they are a multiple of 6 nucleotides in length, termed the 'rule of six' (Calain and Roux, 1993). NDV conforms strictly to the 'rule of six' whereas the replication of

Figure 1.1: Classification of the Mononegavirales virus family. Mononegavirales are negative sense, single stranded RNA viruses, which comprise four virus families.

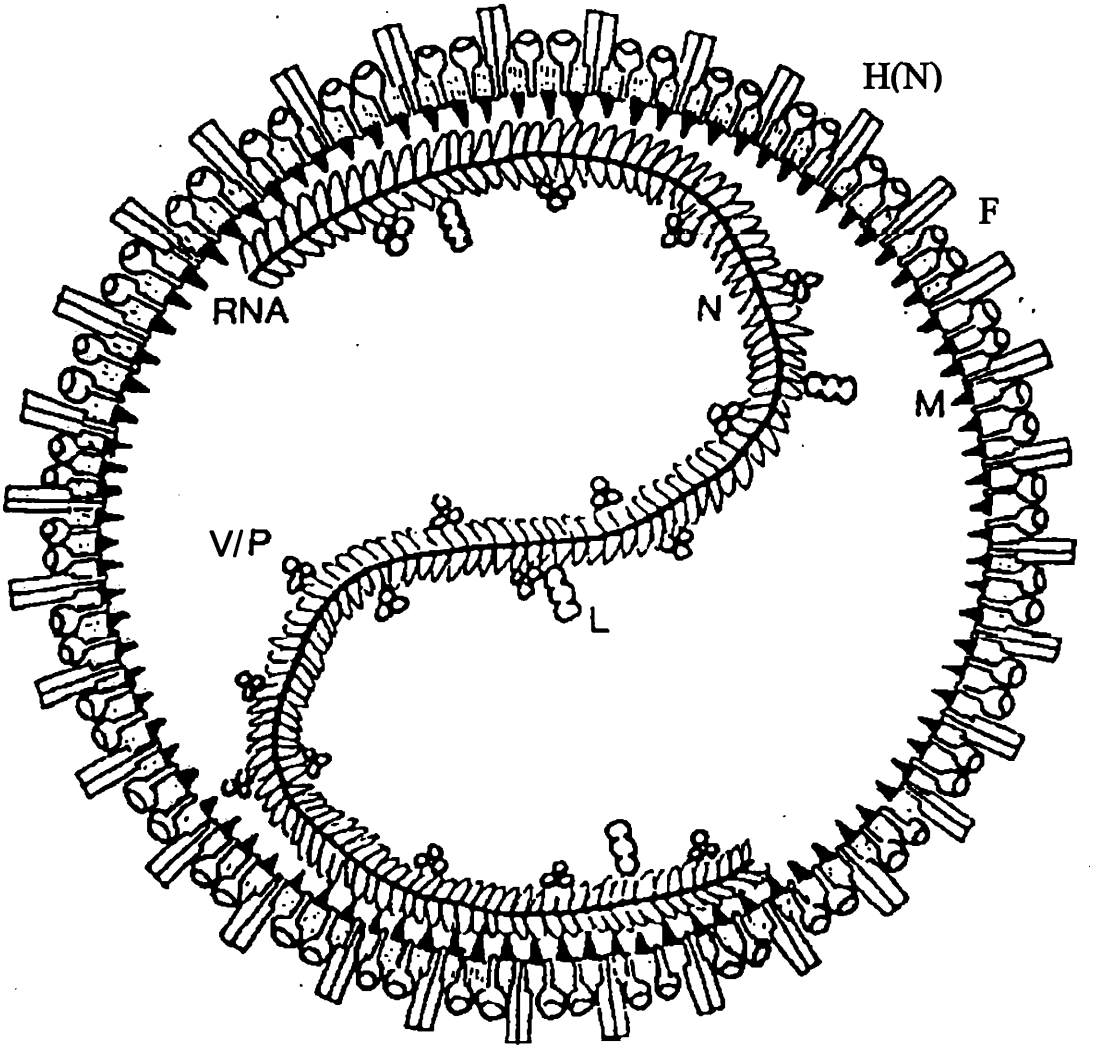


other rubulaviruses are enhanced by this rule but not dependent on it (Murphy., 1997). Thirdly, the mRNA start sites for NDV are clustered over four subunit hexamer phasing positions (Kolakofsky *et al.*, 1998), whereas mRNA start sites of other rubulaviruses cluster over only three positions. Fourthly, anti-human parainfluenza virus 2 (hPIV2) monoclonal antibodies (MAb) react with simian virus 5 (SV5) and MuV but never with NDV (Tsurudome *et al.*, 1989). Finally, the NDV nucleoprotein (NP) does not require the P protein to bind the RNA, unlike SeV (*Paramyxovirus* genus) and hPIV2 (*Rubulavirus* genus) (de Leeuw *et al.*, 1999). Hence, NDV appears to have evolved separately from other rubulaviruses, possibly due to a difference in host, which is avian rather than mammalian.

1.2.2 STRUCTURE

Mumps virions are pleomorphic in shape but usually seen as rough spherical particles under the electron microscope, although filamentous forms can also be observed. The virions are 150nm or more in diameter and are composed of a negative sense RNA helical nucleocapsid associated with the nucleoprotein (NP), a phosphoprotein (P) and a large protein (L). This nucleocapsid complex is associated with the matrix protein (M), which is membrane associated. The virus envelope is derived from the host cell plasma membrane. Protruding from this envelope are two glycoproteins of approximately 8-12nm in length, known as the haemagglutinin-neuraminidase (HN) glycoprotein and the fusion (F) glycoprotein. Some rubulaviruses, of which MuV is one, also contain a small hydrophobic (SH) protein, thought to be membrane associated. The SH protein has only been detected in MuV-infected cells (Takeuchi *et al.*, 1996). Figure 1.2 depicts the structure of a MuV virion. Two mRNA species are transcribed from the MuV P gene; one directing the synthesis of the V protein and one directing the synthesis of the P protein (see section

Figure 1.2: Diagram of a member virus of the subfamily *Paramyxovirinae*. N: Nucleocapsid; V: V protein; P: Phosphoprotein; L: Large protein; M: Matrix protein; H(N): Haemagglutinin (Neuraminidase) protein; F: Fusion protein (Rima *et al.*,1995).



1.3.2). The V protein has been detected in MuV-infected cells and in virions (Takeuchi *et al.*, 1990) but has been shown to be unstable and is gradually degraded in virus infected cells (Hu *et al.*, 1993).

1.2.3 GENOME ORGANISATION

MuVs are single-stranded, non-segmented, negative sense RNA viruses. The relative positions of the different genes on the viral RNA, encoding the different MuV proteins is as follows:

3' - NP - P - M - F - SH - HN - L - 5'

The genome consists of a non-transcribed leader sequence of fifty-five nucleotides and a trailer sequence of twenty-four nucleotides. The intragenic start and stop sequences are separated by intergenic sequences of one to seven nucleotides. The gene lengths, both in nucleotides and amino acids and their respective molecular weight are as shown in Table 1.1.

Table 1.1: The MuV protein lengths, in nucleotides and amino acids and their respective molecular weights (Wolinsky and Waxham, 1990). The nucleotide lengths include non-coding regions.

PROTEIN	NUCLEOTIDE LENGTH	AMINO ACID LENGTH	MOLECULAR WEIGHT (Kd)
NP	1,845	549	69[1]
P	1,312	391	45[1]
M	1,253	375	40[1]
F	1,721	538	65[1]
SH	310	57	6.7[2]
HN	1,887	582	80[3]
L	6,925	2261	180[3]

[1] Merz *et al.*, 1983
[2] Elango *et al.*, 1989
[3] Rima *et al.*, 1980

1.3 THE MUMPS VIRUS PROTEINS

1.3.1 THE NUCLEOPROTEIN

The nucleoprotein (NP) is responsible for the encapsidation of genomic RNA into an RNAse-resistant nucleocapsid of which it is the main component. The NP also associates with the P and L proteins during transcription and replication and probably interacts with the M protein during virus assembly. The intracellular concentration of unassembled NP is thought to be the major factor controlling rates of transcription and replication.

The sequences of many paramyxovirus NP genes have been determined (Morgan *et al.*, 1984; Rozenblatt *et al.*, 1985; Ishida *et al.*, 1986 & Elango *et al.*, 1989), and this sequence data, along with protease digestion data, suggests that the NP protein is composed of two domains. The N-terminal 80% of the NP is relatively well conserved among paramyxoviruses within the same genus. The N-terminal body of the NP is thought to be a globular domain, which has an overall positive charge that increases the affinity of the N-terminal domain for RNA binding. Hence, the N-terminal domain is responsible for RNA binding and protection. Deletion mutant analysis of the N-terminal domain of sendai virus (SeV), a paramyxovirus, showed that this region was required for nucleocapsid assembly (Buchholz *et al.*, 1993). The N-terminal also possesses NP:NP binding activity, presumably essential for ribonucleoprotein (RNP) formation (Liston *et al.*, 1997).

The C-terminal appears to extend as a tail structure from the globular N-terminal. The C-terminal 20% is not well conserved and has a highly negative charge. This tail structure contains most of the protein's phosphorylation sites and antigenic sites (Hsu and Kingsbury, 1982). The function of this tail appears to be concerned with the mediation of P protein binding to nucleocapsids as monoclonal antibodies directed against the C-terminal domain, released P protein units, which are usually tightly bound within the nucleocapsid structure (Ryan and Portner, 1990). In addition, the C-terminal domain was determined to be unnecessary for producing an encapsidated, complementary copy of the

template during transcription, as deletions in the C-terminal domain had no effect on this process whereas deletions in the N-terminal domain eliminated this activity. However, templates assembled with C-terminal deleted NPs were unable to act as templates for new rounds of genome replication. Therefore the C-terminal tail appears to be essential for this function. After cleavage and removal of the C terminal domain, no difference was observed in RNA binding or protection (Heggeness *et al.*, 1981), suggesting that the non-conserved C-terminal domain is not responsible for RNA binding or for protection of the RNA from degradation.

The NP, along with the P and L proteins, combine together to form the nucleocapsid complex, a single stranded, left handed helical structure of about 1μm length. The exact arrangement of the three proteins in the nucleocapsid is unknown. They are flexible structures and can be coiled within the virus particles. The function of the nucleocapsids is to protect genomic RNA and they are the sites of viral RNA synthesis.

1.3.2 THE PHOSPHOPROTEIN

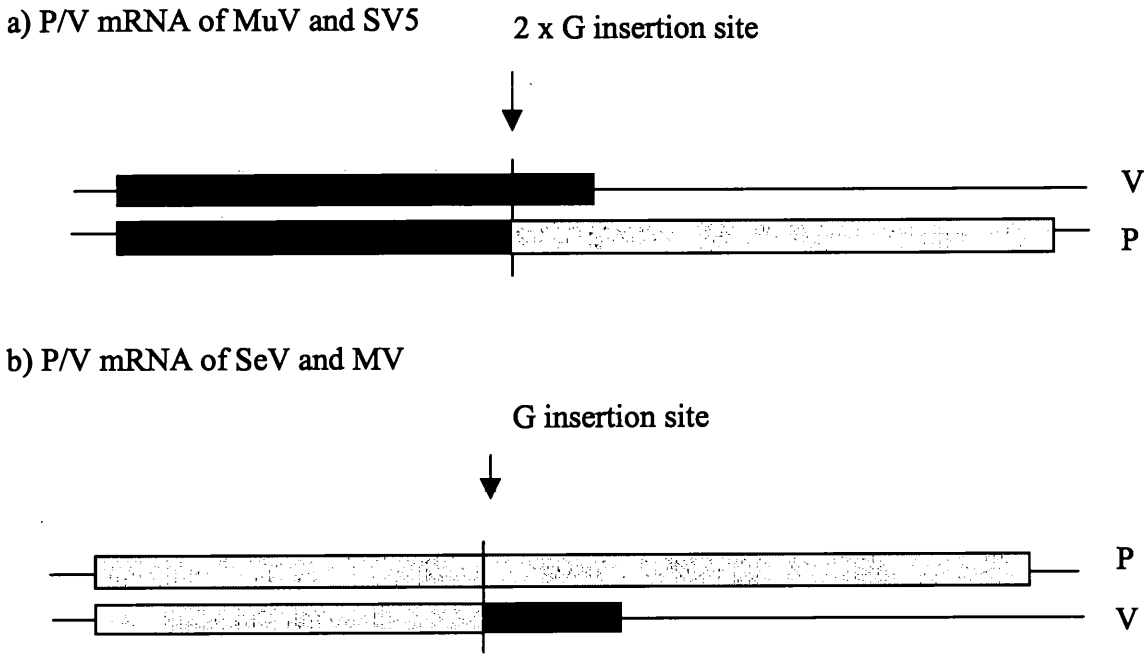
The phosphoprotein (P) is, as its name suggests, highly phosphorylated. It plays a central role in MuV RNA synthesis and with the L protein, it forms the viral polymerase (P-L) and with the unassembled NP, it forms a complex which is possibly the active form in RNA encapsidation (Horikami *et al.*, 1992). The P gene is variable in length within the *Paramyxoviridae* family and within the rubulavirus genus the P gene consists of 245 to 397 amino acid residues.

Transcription of the P gene, except for that expressed within the pneumovirus genus, gives rise to more than one gene product by means of overlapping reading frames and by a process of transcription known as RNA editing, the consequence of which is a reading frame shift. The use of overlapping reading frames is a simple and efficient way for negative strand viruses to extend their genetic information without extending their genome

size. The MuV P gene encodes two proteins, V and P. The unedited version of the P mRNA encodes the V protein and, in rubulaviruses, at the RNA editing site one or two non-coded G nucleotides are co-transcriptionally inserted into the mRNA resulting in a frame shift to yield the P mRNA (Figure 1.3a). The P protein is a fusion of the N-terminal portion of the V reading frame with the second open reading frame. The P and V proteins therefore share the same N-terminal amino acid sequence upstream of the G insertion site. The action of this editing mechanism is unknown but it is thought to occur by polymerase stuttering (Matsumura *et al.*, 1999). This editing mechanism has been observed for other paramyxoviruses such as Sendai virus and is directed by a template stutter site located upstream of the insertion site (Hausmann *et al.*, 1999). The stuttering model proposes that the polymerase pauses at the editing site and nascent RNA slips, causing insertion of extra G nucleotides (Vidal *et al.*, 1990). The V protein is present in rubulavirus virions but its function is unknown. For the paramyxovirus genus, morbillivirus genus and NDV, the V protein is produced from the co-transcriptionally edited P mRNA. Therefore, upon translation the N-terminal half of the P protein sequences are fused to the highly conserved, cysteine rich domain, expressed from the V protein open reading frame (Figure 1.3b). Therefore, RNA editing is not necessary for P protein production.

V^- virus constructs of SeV were observed to replicate as efficiently as the wild type in cell culture and eggs, indicating that the V protein is non-essential (Kato *et al.*, 1997). However, the mutant virus displayed greatly increased gene expression combined with severe cytopathogenicity in some cell lines. This suggests the interaction of the V protein with cellular factors. In mice, the V^- virus produced greatly reduced pathogenicity observed as a normal increase in body weight with minimal lesions in the lungs; the target organ (Kato *et al.*, 1997). These results suggested different *in vivo* and *in vitro* functions of the V protein.

Figure 1.3: The mRNA of the P gene of paramyxoviruses, showing the method by which the P gene is edited to give rise to more than one gene product. In MuV and SV5 the P protein is a fusion of the N-terminal portion of the V protein with the second ORF, whereas, for MV and SeV RNA editing is not required for P protein production.



Studies on SeV (Didcock *et al.*, 1999) indicated that, although SeV does not naturally infect humans, it blocks the interferon (IFN) response in human cells; SV5 was also observed to block the IFN response in human cells. The authors also noted that SeV but not SV5 inhibited interferon response in murine cells. Studies on simian virus 5 (SV5) have concluded that this virus inhibits IFN signalling by targeting components of the transcription complexes that activate IFN (STAT1) (Didcock *et al.*, 1999). Expression of the SV5 V protein, in the absence of other proteins, inhibited IFN signalling and induced the degradation of STAT1. The authors concluded that the V protein inhibits interferon signalling thus allowing the virus to replicate *in vivo*.

The N-terminal portion of the SeV P protein contains regions responsible for binding the L protein and the C-terminal domain contains regions responsible for binding of the NP (Curran *et al.*, 1991). The N-terminal of the P protein contains most of the sites at which the protein is phosphorylated (Vidal *et al.*, 1988) and the immediate N-terminal domain

contains a region essential for RNA synthesis although the mechanism is unclear. This domain also contains a unique region required for RNA encapsidation (Curran *et al.*, 1994). The regions for L and NP binding within the P protein of SeV have been differentiated (Curran, 1998).

The P genes of the *morbillivirus* and *paramyxovirus* genera can be edited to give rise to a third protein product known as the C protein. Rubulaviruses do not express this protein. It is a small protein the function of which is unknown. Using antibodies directed against both P and C proteins of morbillivirus MV it was observed that both proteins were expressed in virally infected cells (Bellini *et al.*, 1985). The C protein localised in the nucleus, suggesting that MV may have an obligate nuclear phase in replication. The P protein localised with cytoplasmic inclusions.

1.3.3 THE MATRIX PROTEIN

The M protein is the most abundant protein in the virion. The nucleotide sequences of many paramyxovirus M genes have been determined (Blumberg *et al.*, 1984, Chambers *et al.*, 1986, Elango *et al.*, 1989, Afzal *et al.*, 1994). The M protein is a basic protein with hydrophobic domains but none large enough to span a lipid bilayer, hence, it is thought to be only peripherally associated with the membrane. The M protein is synthesised in free ribosomes and is located underneath the viral lipid bilayer within the virion.

The main function of this protein is thought to be in the organisation of the virion structure, in preparation for virus budding through the plasma membrane of the infected cell (Yoshida *et al.*, 1976; Matsumoto., 1982). The M protein is thought to associate with the cytoplasmic tails of the envelope glycoproteins, the lipid bilayer and the nucleocapsids. The nucleocapsids have an overall acidic nature whereas the M protein is basic; this may support ionic bonding when the two units associate. The M protein can also associate with itself and this association is thought to be essential for the formation of a budding virus

particle. Analysis of SeV mutants with deletions in the hydrophobic region, resulting in an overall negative charge of the putative amphipathic helix, prevented M protein self-association. In contrast, SeV mutants with deletions in the hydrophobic region, resulting in an overall positive control increased virion formation (Mottet *et al.*, 1996). The M protein of many paramyxoviruses is phosphorylated but the function of this is unknown.

Studies on proteins from other negative sense RNA viruses have provided some insight into the structure of paramyxovirus proteins. For example, the vesicular stomatitis virus (VSV), a member of the *rhabdoviridae*, M protein consists of at least two distinct domains (Coulon *et al.*, 1990). One domain is responsible for structural interactions between the RNP and the lipid bilayer, as well as for the regulation of transcription. The second domain is thought to be responsible for the shut-off of cellular RNA synthesis and for the recognition of a cellular factor required for efficient viral RNA synthesis. It is possible that the MuV M protein shows similarities, in structure and function, to the VSV M protein.

In persistent virus infections, such as SSPE, the MV M protein is produced at normal rates. However, it is unstable and virus budding fails to occur, hence allowing the infection to remain persistent (Hirano *et al.*, 1992). The M protein of a MV strain which caused acute measles associated with the intracellular viral nucleocapsids and the plasma membrane, whereas the M protein of a virus which caused SSPE localised mainly in the cytosol and could not bind viral nucleocapsids. The M protein of the acute measles strain could bind SSPE nucleocapsids. The M proteins of MV strains that cause SSPE exhibit a defect in nucleocapsid-binding function (Hirano *et al.*, 1993).

1.3.4 THE FUSION PROTEIN

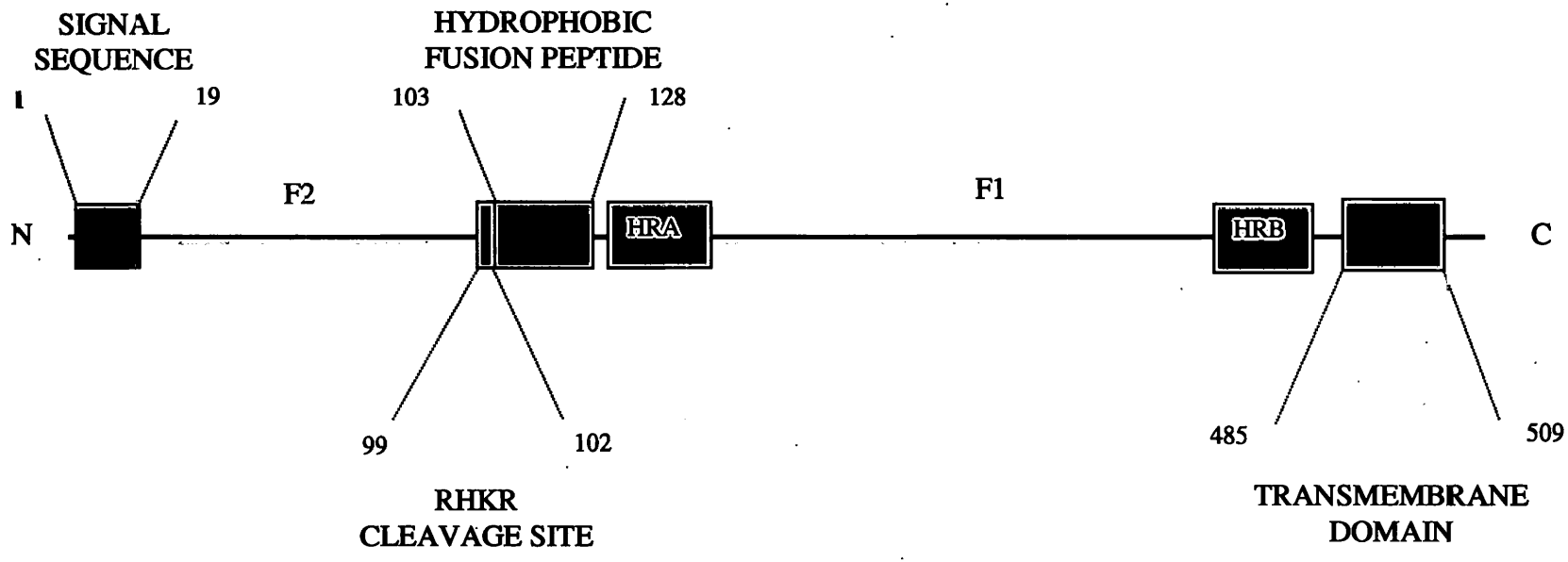
The fusion (F) protein is the major viral glycoprotein involved in virus to cell and cell to cell fusion (Bratt and Gallaher *et al.*, 1969). The F protein is a type I integral membrane protein (its N-terminus is external to the virion), which spans the membrane once. It has a

three domain structure consisting of a large, relatively hydrophilic domain external to the virion, a second domain of approximately twenty uncharged amino acid residues that anchor the protein to the membrane and a second hydrophilic domain on the inner side of the virion envelope. At the N-terminus of the F protein is a cleavable signal sequence which targets the polypeptide to the membrane of the endoplasmic reticulum (ER) during translation. At the C-terminus of the F protein is a hydrophobic domain that acts as the stop transfer domain and is a transmembrane domain, anchoring the protein in the membrane. Figure 1.4 shows a schematic diagram of the F protein.

The F protein is synthesised as an inactive precursor, F_0, which is cleaved by a host cell protease, forming a biologically active protein consisting of two sub-units F_1 and F_2 linked via a disulphide bond (Scheid and Choppin, 1977). The cleavage of the F_0 is vital for infectivity and pathogenicity. Cleavage of the F protein occurs intracellularly during translocation of the protein through the trans Golgi network. Furin, a host cell protease, is located in the trans Golgi network and it is believed to be responsible for the cleavage of F_0 to F_1 and F_2 (Ortmann *et al.*, 1994). Furin is also responsible for processing a number of blood clotting factor and growth factors and has been shown the cleave rat proinsulin in the production of insulin (Smeekens *et al.*, 1992). Strains of NDV that contain multibasic residues at the cleavage site are virulent for their host whereas strains that have only a single basic residue are avirulent (Nagai *et al.*, 1976, Glickman *et al.*, 1988). Cleavage of F proteins with single basic residues does not usually occur in cell culture unless an exogenous protease is added.

Sequencing studies on paramyxovirus F proteins have shown that the N-terminal twenty amino acid residues of F_1 are hydrophobic and highly conserved. These are termed the fusion peptide. The fusion peptide is usually hidden within the protein, but during the fusion process, a conformational change allows the fusion peptide to interact with the cell membrane, initiating fusion. It is believed that the active form of the fusion peptide is an

Figure 1.4: Schematic diagram of the MuV fusion (F) protein. The F protein is composed of two sub-units: F1 and F2. At the N-terminus of F2 is the signal sequence, used to transport the polypeptide to and through the cell membrane. The cleavage site, between the two sub-units is followed by a hydrophobic region, thought to be the fusion peptide. Two HR domains are located within the F1 sub-unit, as is the transmembrane domain.



α-helix within which the conserved hydrophobic residues are located on one face of the helix. The F protein also contains two heptad repeat regions. Heptad repeat region A (HRA) is located to the carboxyl-side of the fusion peptide and heptad repeat B (HRB) is located to the amino-side of the transmembrane domain. Heptad repeat regions can form coiled coil structures of α-helices. The α-helical structure consists of seven residue repeats in a sequence a, b, c, d, e, f and g where residues a and d are large and hydrophobic (Chambers *et al.*, 1990). HRB consists of leucine or isoleucine residues lined up on one face of the helix, at positions a and d, for three or four turns of that helix and is therefore known as a leucine zipper motif (Buckland and Wild, 1989).

The heptad repeat regions of the NDV F protein consist of the following amino acids (Chambers *et al.*, 1986):

HRA: **A**kq**N**aan**I**lr**L**kes**I**aa**T**nea**V**he**V**tdg**L**sq**L**ava**V**gk**M**qqf**V**nd**Q**fnk**T**

HRB (leucine zipper motif): **L**gn**V**nns**I**sn**A**ldk**L**ee**S**nsk**L**dk**V**nvk

These heptad repeat regions may change from one form, in a stable state, to another in a more fusogenic state, thus allowing the fusion peptide to interact with the cell membrane.

This is also the case for fusion proteins of other RNA viruses, such as the influenza HA fusion protein (Chan *et al.*, 1997), the human immunodeficiency virus (HIV) gp41 fusion protein (Joshi *et al.*, 1998) and the Ebola virus gp2 fusion protein (Russell *et al.*, 1994). These fusion proteins all share similarities; they are all homotrimers (Russell *et al.*, 1994; Chan *et al.*, 1997) containing a heptad repeat region adjacent to the fusion peptide and a second heptad repeat region in close proximity to the transmembrane domain (Chambers *et al.*, 1990) and undergo a conformational change to become fusion active. Recently, synthetic peptides have been synthesised of the heptad repeat regions of rubulavirus SV5 to help determine the role of these regions in membrane fusion (Joshi *et al.*, 1998). The synthetic peptides were observed to form a stable six helical structure, thought to be active

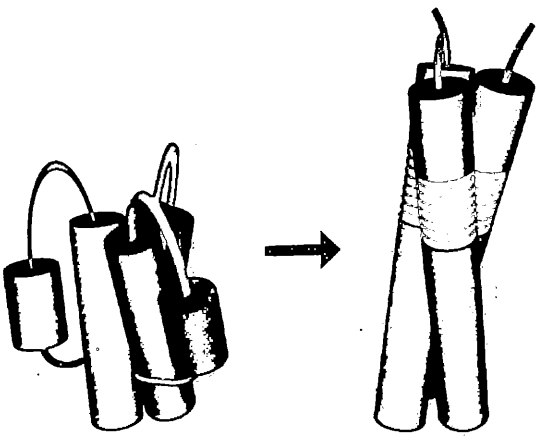
in membrane fusion and the author suggested the F proteins present the fusion peptides to host cells atop a central three stranded coiled coil. Figure 1.5 depicts the structure of the influenza HA fusion protein and the proposed structure of the SV5 F protein. The results of this study by Joshi *et al* (1998) suggest that the F protein of paramyxoviruses can form a similar structure to the influenza HA and the HIV gp41 fusion proteins. This structure of the fusion protein is therefore conserved amongst widely different viruses (Hughson, 1997).

Three-dimensional (3D) models have been suggested, based on low-resolution cryo-electron microscopy for the fusogenic state of HA. Böttcher *et al* (1999) three dimensionally reconstructed intact HA in two distinct conformations, in its native and fusogenic states. Detailed changes were detected in the fusogenic state of HA, the main change being the formation of a continuous central cavity through the trimer. Other data also suggests relationship of a 3-state model to the mechanism of virus fusion by HA (Puri *et al.*, 1990). Here, the HA undergoes a proton driven shift from a tense state at neutral pH, to a metastable state (the fusogenic state). This is followed by a shift to a desensitised state. Böttcher *et al* (1999) concluded that the 3D fusogenic state of their HA resembled the metastable state of the 3-state model.

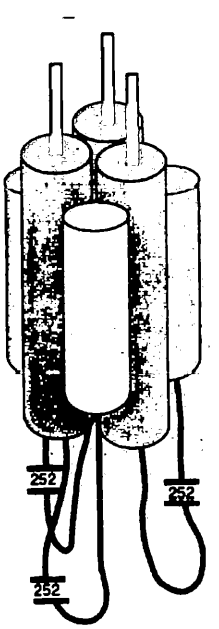
The F protein of MuV has similarities with the influenza virus HA protein, about which considerable information has been gathered. The HA protein of influenza is a membrane bound glycoprotein, synthesised in the rough endoplasmic reticulum (RER) within an infected cell and transported to the cell surface, where it becomes incorporated into the newly synthesised virions. The HA protein exists as a trimer consisting of identical monomers of HA, the structure of which has been determined by x-ray crystallography (Wilson *et al.*, 1981; Weis *et al.*, 1988), in which HA has the appearance of an elongated cylinder and is triangular in cross-section. Each HA monomer is composed of two structurally distinct regions, a triple stranded coiled coil of α-helices and a globular region

Figure 1.5: The proposed structure of the trimeric coiled coils found in the fusion proteins of A) influenza HA (Carr *et al.*, 1993); the native state of HA2 is depicted on the left as a stem of three helical hairpins (red, yellow and pink) fastened by the N-terminal fusion peptide (blue) which was buried in the core of the coiled coil. The fusogenic state of HA2 is depicted on the right where the fusion peptide was released from the interior. HA1 subunits were not shown. B) SV5 F protein (Joshi *et al.*, 1998); the F protein was depicted in its fusogenic state where the fusion peptide (red) was exposed. The α-helices formed by HRA are green and those formed by HRB are blue. The remainder of the F1 subunit is shown as a loop in black. The F2 subunit was not shown.

A)



B)



of ß-pleated sheets containing the receptor binding site (RBS) and variable antigenic determinants. Each HA monomer is initially synthesised as a single polypeptide chain of 550 amino acids, known as HA0. After translation of the polypeptide four types of processing occur. Firstly carbohydrate side chains are added, the number and position of which vary with the virus strain (Wiley and Skehel, 1987), next proteolytic cleavage occurs by which the amino-terminal hydrophobic signal sequence is removed from the newly synthesised HA. Thirdly, the glycoprotein undergoes fatty acid acylation during which palmitic acid is added to cysteine residues near to the carboxy-terminus. Lastly, a specific cleavage by a host cell protease (Klenk *et al.*, 1975) cleaves HA0 into HA1 and HA2 by the removal of arginine at position 329. The two chains are covalently linked by disulphide bonds. This cleavage stage occurs late in the maturation of the glycoprotein and is essential for virus infectivity.

A comparison of the amino acid sequences of *Paramyxoviridae* F proteins does not show much overall homology, but there is similar positioning of glycine and proline residues and the overall hydrophobicity suggests that the structure of the F proteins are similar. Cysteine residues are conserved within the F protein suggesting that the folding of the protein is critical for its function. Which of the cysteine residues act to bond the two subunits together is unknown. The F protein of paramyxoviruses is subjected to glycosylation (Morrison and Simpson, 1980, Lambert *et al.*, 1988) and in MuV there are six to seven possible sites for N-linked glycosylation but as yet there is no evidence as to which of these sites are used. The F1 subunit contains both complex and high mannose oligosaccharides whereas the F2 subunit only contains high mannose oligosaccharides (Herrler and Compans, 1983).

For MuVs, syncytium formation only occurs when both HN and F proteins are expressed (Tanabayashi *et al.*, 1992), suggesting some interaction between the F and HN proteins for syncytium formation. This was also observed for NDV (Morrison *et al.*, 1991), bovine

parainfluenza virus 3 (bPIV-3) (Sakai and Shibuta, 1989) and hPIV-3 (Ebata *et al.*, 1991). However, studies on MV and SV5 showed that the HN, or H protein for MV, had no effect on the fusion ability.

It has been suggested that amino acid at position 195 within the F protein plays a critical role in determining the extent of cell fusion induced by MuV (Tanabayashi *et al.*, 1994). Replacement of Ser 195 by aromatic amino acids, Tyr, Trp or Phe, significantly reduced the fusion ability of the otherwise fusion competent RW strain of MuV. Replacement of Ser 195 by charged amino acids without aromatic side chains did not alter the fusion ability of a virus. The mutant F proteins with Phe, Asp or Arg at position 195 remained uncleaved. Cleavage of the F protein occurs in the Golgi network; hence, the authors predicted that the mutant F proteins were incorrectly folded, disturbing protein transport from the ER to the golgi and so preventing cleavage. It is unknown how the fusion protein interacts with the cell membrane to cause cell fusion but it has been suggested that amino acid at position 195 could have an essential role in establishing a possible interaction with the HN protein (Tanabayashi *et al.*, 1994).

1.3.5 THE SMALL HYDROPHOBIC PROTEIN

The gene encoding the small hydrophobic (SH) protein is located between the genes encoding the F and HN proteins on the RNA of rubulaviruses, MuV and SV5. The SH protein of MuV is a hydrophobic integral membrane protein, expressed at the plasma membrane with the orientation of a type II protein (Takeuchi *et al.*, 1996). The function of this protein is unknown, however, when the SH gene was deleted from an infectious cDNA clone of SV5 infectious virus could be recovered, indicating that this protein was not necessary for virus viability in tissue culture. No change was observed in the phenotype of the SH-deleted mutant (He *et al.*, 1998).

The nucleotide sequences of SH genes are highly variable between strains of MuV and are useful for epidemiological studies. Sequence data of the SH gene for many MuV strains have been analysed (Yeo *et al.*, 1993; Strohle *et al.*, 1996; Afzal *et al.*, 1997; Orvell *et al.*, 1997), and results from computer analysis of the sequence data suggest that MuV isolates form six clusters or genotypes, A to F (Wu *et al.*, 1998; Tecle *et al.*, 1998). Table 1.2 shows the main isolates from each of the six clusters. Antigenic differences have been observed between viruses isolated from these genotypes, where genotype A appears to show the most pronounced difference in neutralising epitopes and genotypes B, C and D share a number of neutralising epitopes (Yates *et al.*, 1996 & Tecle *et al.*, 1998).

Table 1.2: Mumps virus strains are split into six genotypes according to the sequence of their SH genes.

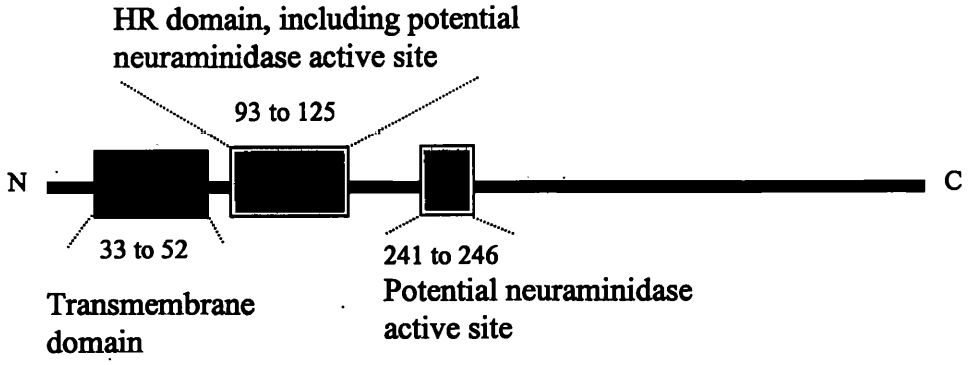
GENOTYPE	ISOLATES
A	SBL-1, JL, RUBINI, ENDERS, CANADIAN AND USA isolates
B	JAPANESE isolates
C	EUROPEAN AND CANADIAN isolates
D	EUROPEAN and CANADIAN isolates
E	UK isolates
F	CHINESE isolates

The sequence data of the SH gene show a highly conserved region of fifty nucleotides, which precede the initiation codon, whereas the open reading frame is highly variable. A high proportion of variability was observed for the first or second base of the codon of the open reading frame (ORF).

1.3.6 THE HAEMAGGLUTININ-NEURAMINIDASE PROTEIN

The HN glycoprotein is a type II membrane protein (its N-terminus is located internally to the virion) which spans the membrane once. It contains a single hydrophobic domain which acts as a signal sequence to translocate the protein across the ER membrane and as a stop transfer sequence to anchor the protein in the cell membrane (Figure 1.6).

Figure 1.6: Schematic diagram of the HN protein showing the transmembrane domain/stop transfer signal at the N-terminus, the position of the HR domain and the position of the putative neuraminidase active sites.



The HN protein is responsible for binding of the virus to the host cell and has an undefined role in cell fusion. The HN protein is assumed to bind to sialic acid containing molecules (Markwell and Paulson, 1980), such as glycoproteins or glycolipids, on the cell surface. In certain paramyxoviruses, this binding is of sufficiently high affinity that these viruses can agglutinate red blood cells of certain mammalian and avian species to cause haemagglutination. As its name suggests, the HN protein also has neuraminidase activity to prevent self-aggregation of the virus particles upon virus budding from the cell and to prevent re-adsorption of newly synthesised virions to the infected cell. The neuraminidase has an acidic optimum pH suggesting that it acts in the acidic trans Golgi network to cleave sialic acid residues from the HN and F chains. There is a lack of understanding about the role of the neuraminidase protein in the entry pathway of viruses. It is thought to catalyse the cleavage of the terminal sialic acid residues at α-2,3- linkages and α2,6-linkages from adjacent sugar residues of glycoproteins and glycolipids to allow the virus to move through the mucous rich areas (Corfield *et al.*, 1981; Paulson *et al.*, 1982).

The paramyxovirus HN protein has some similarities to the influenza NA protein. The influenza NA glycoprotein is also a type II protein and is a tetramer consisting of identical subunits held together by disulphide bridges with a mushroom-shaped appearance under the electron microscope and a box shaped head on the top of a thin stalk (Varghese *et al.*,

1983). This stalk is anchored in the viral membrane by the hydrophobic N-terminal region, which acts as the signal for transportation to the cell membrane. The NA protein is glycosylated, having N-linked oligosaccharides attached to asparagine residues. Four catalytic sites have been identified on the surface of the head. The structures of two serotypes of neuraminidase from influenza A have been determined (N2, Varghese *et al.*, 1983; N9, Baker *et al.*, 1987) and consist of a propeller-like arrangement of six strongly twisted 4-stranded β sheets, this six-fold topology being a novel finding. Two distinct calcium binding sites are located in the tetramer, on the four fold axis between a cluster of eight acidic groups and these appear to aid the stability of the glycoprotein.

As discussed for the influenza NA protein, the MuV HN is an oligomer consisting of disulphide linked homodimers forming non-covalently linked tetramers. It is unknown where the sites for receptor binding, or for neuraminidase activity are and whether the sites are combined or isolated. A hexapeptide, at position 241 to 246, with amino acid sequence NRKSCS, within the globular head of the HN protein is completely conserved among all paramyxoviruses. This region has some homology with the influenza NA protein active binding site which suggests that it may form part of the neuraminidase active site (Jorgensen *et al.*, 1987). A second region, at positions 93 to 110, within the HR domain of the HN protein has also been implicated in neuraminidase activity (Wang and Iorio, 1999). It is assumed that the hexapeptide is more relevant for neuraminidase function due to its homology with the influenza NA protein active site and its sequence conservation amongst the paramxyoviruses. Mutations have been introduced into this region of rubulavirus NDV resulting in decreased fusogenicity, suggesting that the globular head is involved in the interaction with the F protein to induce fusion (Mirza *et al.*, 1994).

The HN protein has two HR domains, between amino acids 93 and 125, which are thought to form α-helical structures that can possibly interact with the HR domains of the F protein (Stone-Hulslander and Morrison, 1999). Mutations in these HN HR domains were

observed to adversely affect fusion. It was hypothesised that when the HN glycoprotein attaches to its receptor it undergoes a conformational change, which induces a corresponding change in the associated F protein, releasing the hydrophobic fusion peptide into the target membrane and initiating fusion (Stone-Hulslander and Morrison, 1997, Tong and Compans, 1999).

Not only is the HN glycoprotein multifunctional but it is also the major antigenic determinant; the protein against which most neutralising antibodies are raised. Monoclonal antibodies are very useful for investigating the characteristics of viral proteins, and several studies have attempted to determine the antigenic sites within the HN protein by selecting and analysing monoclonal antibody resistant (MAR) mutants. A study of the Urabe strain identified amino acid residues 359 (Arg in the mutant, Gln in the wild type) and 335 (Lys in the mutant, Glu in the wild type) as part of an antigenic site within the HN glycoprotein (Yates *et al.*, 1996). A previous study of MAR mutants of the KH strain of MuV identified a region encompassing amino acid residues 352 to 360 to be a putative epitope for the selecting MAb (Kovamees *et al.*, 1990).

The *morbilliviruses* do not encode an HN protein. Instead, they encode an H protein, which has no detectable neuraminidase activity. MV, a morbillivirus, uses CD46 on cell surfaces as the receptor and hence the need for neuraminidase to prevent self-aggregation might not be essential. Pneumoviruses do not contain an HN protein either; instead they encode a G attachment protein. This G protein is also the main antigenic determinant. Epidemiological studies of hRSV and bRSV have focused on the G protein because it shows the highest degree of antigenic and genetic diversity among all the pneumovirus proteins (Martinez *et al.*, 1999; Elvander *et al.*, 1998). The cellular receptor for the *pneumoviruses* is as yet unknown.

1.3.7 THE LARGE PROTEIN

The L protein is the largest and least abundant viral protein encoded by the L gene close to the 5' end of the viral RNA. It is assumed that the L protein, due to its low abundance, large size and localisation to the transcriptionally active viral core, is the viral RNA-dependent RNA polymerase.

The nucleotide and amino acid sequence of many L proteins of the paramyxovirus family have been analysed (Kawano *et al.*, 1991; Blumberg *et al.*, 1988; Higuchi *et al.*, 1992). They are all of a similar length, approximately 2,200 amino acids, but there is little overall homology outside of the subfamilies. However, there are five short regions of homology in close proximity to the centre of the protein, and these regions are also conserved in other RNA-dependent polymerases of other virus families (Poch *et al.*, 1990). These regions are thought to be crucial for the enzymatic function of the protein.

The P and L proteins form a complex (P-L), and both components are required for polymerase activity with the NP:RNA template (Hamaguchi *et al.*, 1988). Transcriptionally active nucleocapsids contain 5 to 10 P proteins per L protein. It is also assumed that the L protein is involved in the addition of the poly A tail to the 3'end and of caps to the 5'end of the mRNA. The methyl-G cap, added to post-transcriptional mRNAs, is assumed to be added by the L protein because transcription and replication occur in the cytoplasm, whereas cellular mRNAs are capped by enzymes located in the nucleus. The large size of this L protein is consistent with its ability to perform these enzymatic functions (Chinchar and Portner, 1981).

1.4 VIRUS REPLICATION

The first step in the infection cycle is entry to the target cell. To gain entry to the cell, the virus must first attach to the cell via an interaction between the viral envelope glycoprotein/s and a host cell receptor/s. A virus receptor may be defined as a macromolecule or complex of macromolecules naturally occurring on the host cell surface which specifically binds the virus and through this binding, starts the infectious cycle of the virus (Markwell *et al.*, 1984). Most paramyxoviruses bind cells via sialic acid residues situated on cell surface glycoconjugates. Sialic acid is the group name for an acyl derivative of neuramic acid and approximately twenty different types of sialic acid have been identified. A sialidase enzyme from the bacterium *Vibrio cholerae* destroys the sialoglycoconjugate receptor and protects the host cell from infection by SeV (Markwell *et al.*, 1980). Hence, the host cell receptor for paramyxoviruses is presumed to be a sialic acid containing molecule, either a glycoprotein or a glycolipid. Sialic acid-containing proteins or lipids are common to most cells, allowing the virus to enter a number of cell types. Respiratory syncytial virus (RSV) is an exception to the above and binds to cellular heparin sulphate, a glycosaminoglycan (GAG) (Feldman *et al.*, 2000). The role of cell surface GAGs in RSV infection is assumed to involve a specific GAG structural configuration (Hallak *et al.*, 2000) and the RSV-GAG interaction facilitates virus attachment and entry.

In contrast, MV is different to other paramyxoviruses as it uses a protein and not sialic acid as a receptor. A MAb, developed against a specific human cell surface protein, was found to inhibit MV infection (Naniche *et al.*, 1992) and precipitated a protein, identified as CD46 (Naniche *et al.*, 1993). CD46 is a type 1 integral membrane glycoprotein found on nearly all nucleated human tissues and cells. CD46 proteins are expressed in the brain strengthening the hypothesis that MV can spread in the brain of SSPE and MIBE patients (Cattaneo and Rose, 1993; Billeter *et al.*, 1994).

As discussed above, influenza viruses also use sialic acid residues for attachment to the cell surface. In humans, the site of infection by influenza A is the respiratory epithelium lining the trachea, and the virus preferentially binds to and infects the ciliated cells of the epithelium rather than the mucous-secreting goblet cells (Edwards *et al*, 1986), which contain sialic acid with an α2,3 linkage. Epithelial cells contain a sialic acid with an α-2,6-linkage, which is less susceptible to the NA (Schulman and Palese, 1977; Sugiura and Ueda, 1980). The receptor binding site (RBS) on the HA protein is a depression on the globular head, which contains several conserved amino-acid residues and is surrounded by variable immunogenically important amino acids forming epitopes. There is a second, but weaker, sialic acid binding site located at the base of the globular head at the interface between HA1 and HA2.

After MuV has attached to the cellular receptor, the viral membrane fuses directly with the host cell plasma membrane at a neutral pH. This fusion event occurs through the interaction of the viral F protein with the cell plasma membrane, leading to release of the viral nucleocapsids into the cell cytoplasm. It is thought that the F protein has to undergo a conformational change to become activated and cause fusion allowing the usually hidden fusion peptide to interact with the cell membrane. For those paramyxoviruses, which require both HN and F proteins for fusion, such as MuV, this conformational change is speculated to occur through interaction of the HN protein with the F protein.

The mechanism through which membrane fusion occurs has been studied in detail for influenza virus (Klenk *et al.*, 1975). After influenza virus attachment to sialic acid residues on the cell surface, it is internalised by receptor mediated endocytosis in clathrin coated pits, rather than by membrane fusion at the cell surface, as for paramyxoviruses. Surface bound influenza virus is quickly endocytosed, with a half-life of 10-15 minutes and transported to lysosomes where the acid pH causes fusion after an irreversible conformational change in the HA. Only HA which has been cleaved from HA0 to HA1

and HA2 can partake in this fusion event. The influenza virus entry pathway is of considerable interest as a target for antiviral drugs. One such drug, amantadine, at concentrations >0.1mM, indirectly inhibits the activation of the membrane fusion activity of influenza virus HA involved in endocytosis (Hay *et al.*, 1985). The drug is thought to block the ion channel activity of the influenza virus membrane protein M2 so causing the pH of the lysosome to increase and preventing the low pH induced conformational change of HA (Hay *et al.*, 1985).

After virus entry into the host cell and release of the viral nucleocapsid into the cytoplasm, the next stage in virus replication is transcription of the negative sense genomic RNA into mRNA. Associated with nucleocapsids released into the cell cytoplasm are multiple copies of the P-L polymerase. These complexes are scattered throughout the length of the RNP but can only initiate transcription from the 3' end of the RNA.

The genomes of paramyxoviruses and rhabdoviruses are organised as a single transcriptional unit with only one promoter. In rubulaviruses the first gene is separated from the 3' end of the viral genome by a leader sequence of 50 nucleotides, where the first twelve nucleotides are conserved throughout each genus. The rest of the leader sequence is rich in A and U sequences. After transcription of the leader sequence the viral polymerase terminates and reinitiates at the beginning of the first gene.

The frequency with which the polymerase reinitiates transcription after each termination sequence is not 100% in all cases. Therefore, there is a higher proportion of transcripts of the NP and P mRNAs, as compared to the L protein mRNA which is furthest downstream and transcribed last. This gradient of transcripts is one way in which the virus can control mRNA production. Another mechanism involves the inefficient termination of a transcript leading to the production of bicistronic mRNA where only the upstream ORF is translated. This is the case when measles virus down-regulates M gene expression during long term persistent infections, such as SSPE (Cattaneo *et al.*, 1987; Hirano *et al*, 1993). A further

mechanism of control of mRNA production involves genes with overlapping mRNA. For respiratory syncytial virus (RSV), the start sequence of the L gene is situated upstream within the M2 gene. Most transcripts that initiate transcription of the L protein terminate when the polymerase reaches the termination sequence for the M2 protein. Hence, functional L mRNAs are only produced occasionally when the polymerase reads through the M2 termination site (Collins *et al.*, 1987).

The accumulation of viral proteins, translated from the mRNAs initiates the switch from transcription to replication. The switch from transcription to replication has been extensively studied for VSV. Wertz *et al* (1994) demonstrated that the extent of terminal complementarity, rather than the exact sequence of the genome, is a major determinant of whether the template directs transcription or replication. In order to determine the cis-acting sequences necessary for RNA replication, Pattnaik *et al* (1995) constructed plasmids with a series of internal deletions in the DI cDNA. The data determined showed that signals necessary for RNA replication are contained in the 5'-terminal 36 nucleotides and the 3'-terminal 51 nucleotides of the DI RNA genome. Whelen & Wertz (1999) examined sequences of the 3' leader and 5' trailer for roles in transcription and replication by observing effects of alterations to the termini of subgenomic replicons, or infectious viruses. The authors concluded that distinct elements in the leader sequence were required for transcription but not for replication and abolishing the use of the leader sequence as a promoter for transcription enhanced replication. The leader sequence therefore is a less efficient promoter for replication that the trailer sequence, possibly because of its essential role in transcription.

Replication of the negative sense genome occurs via a full-length positive sense intermediate. To produce the full-length positive sense antigenome, the polymerase transcribes the genomic RNA but reads through the gene junctions. It is not clear how the viral polymerase ignores termination signals. It could be caused by the synthesis of NP

and RNA occurring concomitantly (Kolakofsky and Ortin, 1991). Initially, when levels of unassembled NP are low, the polymerase preferentially engages in mRNA synthesis to raise levels of all virus proteins. Later, when levels of unassembled NP are high, the polymerase preferentially engages in genome replication. Genomic RNA synthesis from the antigenome is thought to occur in the same way as antigenome synthesis.

Defective-interfering (DI) particles are common products of MuV replication and are implicated in establishment and maintenance of persistent virus infections (Huang and Baltimore, 1970). During viral RNA replication, DI genomes arise as a result of replicative errors, where the polymerase creates internal deletions and hence, DI genomes are very variable in length. The deletions in the RNA genome may arise when the viral polymerase detaches from the RNA and either rejoins the RNA at a different point or attaches to the nascent strand. The generation of SeV DI genomes has been extensively studied (Leppert *et al.*, 1977 & Amesse *et al.*, 1982), and DI genomes have been observed to arise for two main reasons. A complementary copy of the 5' end replaces the 3' end of the virus genome; this complementary sequence being identical to the 3' end of the antigenome template in replication; these DI genomes are known as 'copy-back' DI genomes. The second type of SeV DI genomes are known as 'fusion' DIs. Fusion DIs are capable of encoding the fusion protein with the amino-terminal of the NP protein and the carboxy-terminal of the L protein. These fusion DIs contain internal deletions but retain both termini of the genome. Defective-interfering viruses cannot replicate without the assistance of the infectious parental virus. Therefore, propagation of DI virus is optimal at a high multiplicity of infection (MOI) when all cells contain an infectious virus genome. Defective-interfering particles are composed of identical constituents apart from their RNA and deprive infectious virus of components for its own multiplication. Hence, the yield of infectious virus is significantly lowered. Propagation of virus at a low MOI results in production of less DI particles.

Addition or deletion of single nucleotides in SeV cDNA prevented efficient replication (Calain *et al.*, 1993). Replication was resumed when further nucleotides were added or deleted but only if the total length of the RNA was divisible by six. This "rule of six" is explained by each NP unit being associated with six nucleotides (Egelman *et al.*, 1989). NDV strictly conforms to this "rule of six", but some other rubulaviruses do not (Murphy and Parks, 1997).

Nucleocapsid assembly occurs in the cytoplasm and results in the production of the helical RNP complex, as observed by electron microscopy (Kolakofsky *et al.*, 1998). After assembly of the RNP complex, the association of the P-L complex occurs. HN and F protein maturation occurs in the ER and only correctly folded and assembled proteins are transported out of the ER and through the Golgi apparatus. Here the oligosaccharide side chains can be modified and cleavage of the mature F protein into the two active subunits occurs. The HN and F proteins are then transported to the cell plasma membrane for budding. Here, it is assumed that the envelope glycoproteins make important contacts with the matrix protein and through this to the nucleocapsid. Budding of the nucleocapsid complex and the matrix protein then occurs through the plasma membrane containing the viral glycoproteins to release mature virions. The neuraminidase activity contained within the HN protein acts to allow efficient release of the virus preventing self-aggregation of the virus particles and re-adsorption to the cell.

1.5 VIRUS PATHOGENICITY

Natural MuV infection is acquired and spread through droplets (Johnson and Goodpasture, 1935). Therefore, the virus needs close-living communities to cause an epidemic (Gordon and Heeren, 1940). After initiation of infection, the virus primarily replicates in the mucosal epithelium of the upper respiratory tract before spreading to the draining lymph nodes (Feldman *et al.*, 1989), followed by a viraemia (Kilham *et al.*, 1948) and infection of

the parotid gland, which does not always occur (Kilham *et al.*, 1949). Infection of the parotid gland involves the ductal epithelium and is accompanied by periductal interstitial edema and a local inflammatory reaction. This inflammation and swelling gives rise to the classical clinical symptoms of MuV infections. Virus can also spread to and infect other organs including the CNS. The virus can spread to the kidneys where it infects epithelial cells of the distal tubules and ureters. This can lead to a viruria, virus being shed in the urine of the infected patient for up to 14 days after onset of clinical symptoms (Utz *et al.*, 1957). There is evidence that the virus invades the CNS possibly by crossing the chorioid plexus which is the primary site of replication following infection of newborn hamsters (Wolinsky *et al.*, 1976), and at least half of all MuV infections are associated with replication of the virus in the CNS. Neurological manifestations during mumps are usually limited to those arising from meningeal infection. Mumps meningoencephalitis is rarely fatal, and most cases resolve without sequelae, although instances of deafness have been reported. Other organs that can become affected by mumps virus infection include the testes, liver, pancreas, spleen, thyroid, kidneys, eyes, thymus, heart, mammary glands, lungs, bone marrow, joints, epididymis, prostate and ovary.

It has been suggested that the attachment proteins of MuV, the HN protein, and of MV, the H protein, have a leading role in determining virus pathogenicity (Love *et al.*, 1985; Liebert *et al.*, 1994). Monoclonal antibody mutants selected using anti-HN MAbs, grown in hamster for MuV and rat for MV, had altered affinity for neurones in the brains. The monoclonal antibodies were raised against the HN protein so it was suggested that differences in this protein were responsible for the change in pathogenicity.

1.6 CLINICAL SYMPTOMS AND DIAGNOSIS

The incubation period, after the first exposure to MuV but before symptoms occur, is approximately 18 days (Henle *et al.*, 1948, Johnson *et al.*, 1935). However, one third of all infections have been reported to be asymptomatic (Philip *et al.*, 1959). Virus can be detected in the saliva several days before the onset of clinical symptoms and up to 5 days after the onset of the clinical disease (Chiba *et al.*, 1973).

The most common clinical feature of mumps virus infection is parotitis, or swelling of the parotid glands which occurs in 95% of all symptomatic cases (Philip *et al.*, 1959). Enlargement of glands is usually maximal at 48 hours when pain increases and the swollen gland can remain inflamed for one week or longer.

In males, MuV infection can give rise to orchitis in up to one quarter of cases. This complication is more common in young males infected after the onset of puberty (Philip *et al.*, 1959). Orchitis involves painful swelling of the testes and can lead to the onset of testicular atrophy of the infected gland. Rarely this leads to sterility (Shulman *et al.*, 1992). Analogous to this, females infected with MuV can suffer mastitis and oophoritis (Philip *et al.*, 1959), and virus can be detected in breast milk (Kilham, 1951). Infection by mumps virus can also lead to CNS involvement. The frequency of CNS involvement was noted as 11% in a St. Lawrence Island epidemic in 1956-1957 (Philip *et al.*, 1959) and 17% in a study of mumps in university campuses (Sosin *et al.*, 1989). CNS involvement as meningitis usually occurs 5 days after the onset of parotitis, commencing with symptoms such as fever, vomiting, neck stiffness, headache and lethargy (McDonald *et al.*, 1989).

1.7 IMMUNE RESPONSE TO MuV

Neutralisation assays, using polyclonal sera, determined that MuVs are monotypic viruses (Cantell., 1961), and re-infection by MuV, following natural infection, is rare. The immune response to a developing MuV infection is complex and can be divided into cellular and humoral responses. The exact time course of the development of specific cellular immunity following infection is not completely understood. The lymphocyte response leads to pathological changes including inflammation of MuV infected tissues. This inflammation is well established at the onset of clinical symptoms, suggesting that specific cellular responses develop during the incubation period. T lymphocytes and specifically sensitised cells in the blood and cerebrospinal fluid (CSF) of some patients with active mumps meningitis are also detected (Nagai *et al.*, 1983).

Cytotoxic T cells have been observed following natural MuV infection, with peak responses detected 2 to 3 weeks after inoculation or immunisation (Chiba *et al.*, 1982). Cytotoxic T cells, restricted to autologous infected target cells, have also been observed in the blood of patients during the first weeks of mumps meningitis (Kreth *et al.*, 1982). MuV specific cytotoxic T cells have also been detected in the CSF of several patients. However, there is little evidence that the development of CMI against MuV is necessary for mumps resolution, as examples of persistent or atypical mumps infections in the T cell immunocompromised have not been reported.

Pre-exposure and post-exposure sera of vaccinated children have been examined, and a correlation established between serum neutralising antibody titres and clinical protection (Goncalves *et al.*, 1999). Children with a serum neutralisation end point (NEP) of 4 or lower were found to be susceptible when naturally exposed to wild type virus. Those with a NEP of 6 or above were observed to be immune when exposed to the wild type whilst those with an NEP between 4 and 6 were classed as equivocal. Results of enzyme immunoassays (EIA) showed no correlation with the NEP results or with clinical

observations. However, the NEP and EIA methods measure different properties of the virus; NEP measures antibody against a biological property (live virus particles) whereas EIA measures antibody against total viral antigen (the amount of HN present, whether or not part of a live virus).

MuV specific IgM responses usually exceed IgG responses early in infection but decline over a six month period. Virus-specific IgM levels are therefore the most useful indicator of a recent mumps infection. Virus specific IgG responses do not peak until 2 to 3 weeks into the clinical infection and persist indefinitely (Meurman *et al.*, 1982). Neutralising monoclonal antibody, against the HN protein, can protect animals from challenge by lethal virus concentrations even if it is administered several days after virus inoculation (Wolinsky *et al.*, 1985). Protection appeared to be the result of the anti-HN MAb limiting the spread of infection within the CNS. IgA responses are detected but only early in the course of the infection.

1.8 ANIMAL MODELS

Several attempts have been made to develop animal models for paramyxoviruses, the most successful to date being the study of MV in rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. Other attempts have involved the use of the hamster and rat for studying MuV and the use of chimpanzees and Cotton rats to study human parainfluenza virus (hPIV) and human respiratory syncytial virus (hRSV). The use of non-human primates as animal models is limited due to availability, expense and ethical considerations, so the alternative of developing an animal model using smaller mammals, such as the hamster for studying MV and CDV and rats for studying MuV, RSV hPIV3, needs attention.

Man is the only natural host for MuV. However, the monkey, dog, cat, ferret, rabbit, guinea pig, adult and suckling hamster, suckling mouse and suckling rat have all been

experimentally infected. A MuV, originally isolated from human breast milk, was adapted to newborn hamster brains, and the resultant virus strain produced meningoencephalitis after intracerebral inoculation of hamsters (Kilham and Overman, 1953). This hamster-neuroadapted virus strain (Kilham strain) has since been used by a number of workers to investigate factors that contribute to neurovirulence because it is a good animal model for this manifestation. The adapted virus was used to determine the mode of spread of the virus to the CNS after intraperitoneal inoculation into hamsters. Pathogenesis of MuV infection in the hamster can be compared to MuV infection of humans as in both cases viraemia and viruria occur, as does the spread of MuV to the CNS (Wolinsky *et al.*, 1976).

Recent studies have suggested that the neonatal rat may be a good model of MuV infection (Rubin *et al.*, 1998). When one-day-old rats were inoculated with either the attenuated JL vaccine strain or the neurovirulent Kilham (KH) strain, histopathological differences could be seen in the rat brain and titres of KH significantly exceeded those of JL. The weight of the rats also differed, with the KH infected rats losing weight but the JL infected rats remaining the same as the controls. The greater neurovirulence of the KH strain was apparent under histological examination of brain material, where hydrocephus and cerebellar abnormalities were seen for the KH infected rats. However, none of the infected rats developed encephalitis. Further work needs to be carried out using other MuV strains to observe whether the rat model can differentiate between other strains of mumps virus because the KH strain of MuV is adapted to growth in rodents, whereas JL is not, and this factor could account for the differences observed in the study.

Prior to the widespread use of the MuV vaccines, MuV was the leading cause of virus-induced central nervous system disease. Problems with neurovirulence of the MuV vaccines stem from the lack of an animal model capable of determining the potential for neurovirulence. Neurovirulence testing of MuV vaccines is currently performed in monkeys but these tests do not differentiate between neurovirulent and non-neurovirulent

strains effectively. The monkey neurovirulence test (MNVT) is required by the World Health Organisation (WHO) and other control agencies to enable evaluation of a seed virus and vaccines derived from it. The MNVT involves at least 20 monkeys, 10 for the test virus and 10 for a reference virus. A 0.5ml inoculum is injected into the thalamus and the infected animals are observed for clinical signs of disease over 7 to 21 days. The animals are then sacrificed under general anaesthetic, the formalin perfused brain is investigated, and specific histological lesions seen are graded on a score of 1-4. Grade 1 is minimal infection, grade 2 is moderate, grade 3 is severe and grade 4 is overwhelming infection where severe inflammatory infiltration occurs (Maximova *et al.*, 1996). In a recent study (Afzal *et al.*, 1999), vaccine strains and a wild type were inoculated into the thalamus of *Macaca fasicularis* monkeys. No strain was observed to produce significant clinical signs. The histological data presented did not distinguish between related strains of virus but did differentiate between vaccine and wild type strains. This study called into question the ability of the MNVT to differentiate between acceptable and unacceptable vaccine seeds and the value of the monkey as a model for MuV infection.

1.9 EPIDEMIOLOGY

MuV is a geographically unrestricted disease with the exception of remote islands and tribes. A study of such tribes (Black *et al.*, 1974) showed that antibodies to MuV were either absent, were present at a low frequency or found only in persons over a specific age. It was postulated that MuV was present in small tribes only on introduction from larger communities after which it spread rapidly but could not maintain itself. In urban populations, mumps occurs endemically, with peak incidences in winter and spring months (Modlin *et al.*, 1975). Cases are usually observed in school children where close contact occurs among susceptibles.

The incidence of mumps has been decreasing since 1941 when there were 250 cases per 100,000 people. At the time of introduction of the first MuV vaccine in 1967, cases were 76 per 100,000 people. Since then, cases of mumps have steadily declined with 1 case per 100,000 recorded by the CDCP in the USA in 1992. However, data of the incidence of mumps provide only a rough idea of the whole picture, as 30% of all mumps infections are considered asymptomatic.

Natural infection by MuV appears to give life-long immunity. Antigenic differences have been reported between MuV isolates but these do not seem to have any epidemiological significance because once acquired, immunity to mumps is valid world-wide. A study of Dutch families detected antibody to MuV in approximately 95% of young adults (Wagenvoort *et al.*, 1980). The remaining 5%, when exposed to the virus, proved to be immune.

Epidemics in isolated populations without prior immunity have been useful in studying MuV epidemiology. An epidemic on St. Lawrence Island in the Bering Sea in 1957 was thoroughly studied (Meyer *et al.*, 1962). Only those under 50 years of age were included in the study as some prior immunity was observed in the older members of the community. Ninety-three percent developed mumps complement fixing antibody but only 70% had recognisable clinical symptoms. The majority of cases occurred in children under the age of 10. Ninety-five percent of symptomatic cases showed parotitis. Orchitis was observed for all ages of males infected, from 3 to 51 years but was more common in those post puberty. Thirty-eight percent of men above 15 had orchitis, and 5% of orchitis patients had no signs of parotitis. Neurological symptoms were observed in 11% with 4 cases of encephalitis and 40 cases with a stiff neck. In the St. Lawrence Island epidemic, long incubation periods were observed where in 95% of cases the incubation period was between 14 and 21 days. Children were the most at risk from acquiring mumps but the incidence of mumps encephalitis increased with age.

1.10 VACCINES

At least 10 different mumps vaccines are in use throughout the world and Table 1.3 summarises the main MuV vaccines in use and their passage history. The vaccine against mumps is administered either as a monovalent mumps vaccine or in a trivalent form known as the MMR vaccine, the other components being vaccines against measles and rubella. The mechanism of attenuation of the live mumps vaccine is unknown, as is the length of protective immunity after vaccination, although it is thought that the vaccines may not confer life long immunity.

Table 1.3: Summary of the main MuV vaccines in use.

VACCINE STRAIN	PASSAGE HISTORY	REFERENCE
UrAm9	Chick embryo amniotic cavity, QEF, amniotic membrane of hens' eggs	Yamanishi *et al* (1973)
Jeryl Lynn	Embryonated hen's eggs, CEF	Hilleman *et al* (1968)
Rubini	WI-38 cells, MRC-5 cells	Gluck *et al* (1986)
Leningrad-3	Chick embryos, Japanese QEF	Smorodintsev *et al* (1965)
Sofia 6	Guinea-pig kidney primary cells	Odisseev and Gacheva (1994)

QEF = Quail embryo fibroblasts
CEF = Chick embryo fibroblasts

The first live attenuated vaccine was produced by seven passages of a wild type mumps virus in the chicken embryo amniotic cavity followed by ten passages on chick embryo fibroblast cells. This vaccine strain is known as the Jeryl Lynn strain (Buynak and Hilleman, 1966), named after the child from whom the virus was isolated. The Jeryl Lynn strain has been shown to be composed of two sub-populations of virus, known as JL-2 and JL-5 (Afzal *et al.*, 1993). The components can be distinguished by their plaque morphology on Vero cells and genetically. This vaccine has been in use since 1967 and

clinical protection studies have shown it produces 95% seroconversion (Hilleman *et al.*, 1968). A second live attenuated mumps vaccine, the Urabe Am9 strain (Yamanishi *et al.*, 1973), was introduced in 1973 into Canada, UK and Europe and Japan. This vaccine was produced from a wild type isolate by two passages in the chick embryo amniotic cavity, four passages in African Green monkey kidney cells, further passaged in embryonated hens' eggs and five passages in quail embryo fibroblasts. Finally, the virus was passaged four times in the amniotic cavity of hens' eggs. The Urabe vaccine causes mild vaccine associated meningitis (Forsey *et al.*, 1990) in up to 1 in 3,800 vaccine recipients (Balraj and Miller, 1995). This resulted in its use in Canada and the UK being terminated. The Urabe vaccine continued being used in France as it provides a higher seroconversion rate of up to 97.8%. The Jeryl Lynn strain has not been implicated in any case of vaccine associated meningitis and is routinely used in the UK, although it may give a lower seroconversion rate than the Urabe strain. Both the Urabe and Jeryl Lynn vaccine strains have been implicated in vaccine associated parotitis in a minority of patients, estimated at approximately 0.7% (Fujinaga *et al.*, 1991).

Other vaccine strains include the Leningrad-3 strain used in Russia (Smorodintsev *et al* (1965)), Sofia 6 used in Bulgaria (Odisseev & Gacheva, 1994) and the Rubini strain (Gluck *et al.*, 1986), which is in use in Switzerland, Portugal, Spain and in some developing countries. The Rubini vaccine was isolated from the urine of a child with mumps and was attenuated by two passages in WI-38 cells, thirteen passages in hens' eggs, and finally the virus was adapted to MRC-5 cells. Recently, investigations have suggested that the Rubini vaccine strain gave less protection in vaccinated individuals as outbreaks have occurred in Portugal (Afzal *et al.*, 1997), Western Switzerland (Germann *et al.*, 1996) and Singapore (Goh *et al.*, 1999).

MuV vaccine strains have mutations in their HN envelope glycoprotein as compared to their progenitor wild type. For example, the Urabe vaccine contains a mixture of two

variants differing at certain positions within the HN gene, one of them at nucleotide position 1081 (Afzal *et al.*, 1998; Brown and Wright, 1998). One variant had an A at this position, which translates to a Lys at amino acid position 335 and the other variant had a G at this position, which translates to a Glu at amino acid position 335. It was suggested that Lys335 was the disease associated form of the virus because it escaped neutralisation by a monoclonal antibody, in contrast to Glu335, which was associated with attenuation. These results show that vaccine associated meningitis in recipients of the Urabe vaccine was not caused by reversion to the virulent type but by a variant of the vaccine strain, present in the vaccine (Brown and Wright, 1998). The results also indicate that a vaccine composed solely of the Glu335 virus would be safer than the existing Urabe vaccine.

There has been a dramatic decrease in mumps incidence in countries where an effective vaccination programme exists. After vaccination was introduced in the UK in 1988, a drop of 79% was recorded by the PHLS between 1989 and 1990. Recent studies have recommended re-vaccination with the MMR vaccine to provide a booster reaction (Tischer and Gerike, 2000, Broliden *et al.*, 1998). Sweden commenced a two-dose programme of MMR vaccination in 1982 to be carried out at the ages of 18 months and 12 years. In 1992-1993 the first group vaccinated at 18 months reached the age of 12 years. Before and after receiving the second dose neutralisation antibodies were tested (Broliden *et al.*, 1998), and it was concluded that a booster vaccine was necessary because the level of neutralisation antibody had waned in the previously vaccinated individuals.

1.11 VIRAL QUASISPECIES AND ADAPTATION

Mumps virus is a negative sense, single-stranded RNA virus. The error rate of eukaryotic viruses has been extensively reviewed by Smith and Inglis (1987). Error rates of viruses have been measured using both direct and indirect techniques. By directly measuring the *in vitro* error rate of the polymerase that is responsible for virus genome replication, by

measuring the frequency of nucleotide misincorporation during transcription, estimations of error rates have been made. This error rate has been estimated at between $10^{-2.4}$ and $10^{-2.8}$ for the reverse transcriptase encoded by retroviruses. The error rate of the VSV RNA polymerase has also been directly determined by measuring the proportion of transcripts of defective interfering particle RNA, no longer susceptible to cleavage by ribonucleotide T1 at a particular site. The error rate was determined to be approximately $10^{-3.15}$. Nucleotide substitution frequencies have also been measured by direct nucleotide sequencing of virus clones derived from the same plaque. Using this technique, frequencies of $10^{-4.1}$ for the influenza NS segment and $<10^{-5}$ for the poliovirus type 1 VP1 gene have been obtained. Nucleotide substitution frequencies have also been measured using indirect techniques, such as by analysis of MAR mutants in a cloned virus stock (Smith and Inglis, 1987).

This genetic diversity is known as quasispecies (Eigen *et al.*, 1981) and viral quasispecies is mainly caused by the infidelity of genome replication, large population sizes, recombination and reassortment. Within a viral quasispecies population, variants exist with unique biological properties and therefore, potential exists for modified forms of diseases to arise and emergence of new diseases. Such individual variants have altered genotypic and may have altered phenotypic characteristics when compared to the consensus virus population but which only become apparent if the variant outgrows dominant phenotype of the virus population. Any change in environmental conditions, by which the variants in a virus population are given the opportunity to compete favourably with the consensus virus population, can shift the ratio of variant to parent. Factors that are included as environmental conditions encompass growth of a virus in alternate host cell lines or hosts, interference by defective interfering particles, immune selection and temperature shifts. The quasispecies nature of RNA virus populations complicates vaccine design because of the potential antigenic diversity. Therefore, vaccines against RNA virus

populations need to be designed to encompass a broad range of antigenic types and live vaccines have the advantage over inactive ones by being of a live quasispecies nature.

Viral quasispecies facilitates adaptation to and improved fitness in existing or changing environments through natural selection. Some of the variants may have a selective advantage over the predominant population, which could enable a variant to become dominant under different environmental conditions of which host cell type may be an example. After one passage in a cell line, which exerts a strong restrictive pressure and in which the predominant population is not able to attach, penetrate or replicate, a single variant may be able to do so; hence, the host cell has selected the variant. If the host cell exerts a weaker selective pressure, then the variant might only slowly become dominant or may remain at the same titre as the predominant population.

A variety of cell lines have been observed to exert pressures that affect the phenotype of RNA viruses. For example, when two related sub-populations of a picornavirus, foot and mouth disease virus (FMDV), were subjected to growth-competition experiments in BHK-21 cells (Sevilla *et al.*, 1998), one sub-population was observed to have a selective advantage over the other population but only when the competition passages were carried out at a low MOI. At a high MOI both sub-populations coexisted. These results suggest that small differences in the virus-cell interaction can contribute to an MOI dependent selective advantage of one viral sub-population over a related one. A second example is that of rhabdovirus VSV fitness on different cell lines (Novella *et al.*, 1995). Following persistent replication in insect cells, VSV increased in fitness, in comparison to the decrease in fitness observed after replication of VSV in mammalian cells. Fitness is measured by the ability of virus to produce infectious progeny.

Cell passage of MuV has been observed to select variants with a different antigenic pattern compared to the previous, predominant population (Yates., 1995). A Vero adapted Urabe vaccine strain of MuV was neutralised by a panel of anti-HN MAbs, whereas the

commercial bulk Urabe vaccine, adapted to CEF cells, was not neutralised by one of the MAbs. Sequencing of the HN gene revealed one amino acid difference at position 335. In the CEF isolate, this was a Lys and a Glu in the Vero adapted virus. The authors also observed that passage in Vero cells appeared to select either a virus with a Lys, instead of an Asn, at position 464 or a Glu, instead of a Lys, at position 335, but not both. Therefore, amino acids 335 and 464 were implicated in host cell selection. A second study by Yates *et al* (1996), also showed the selection of MAR mutants from the Urabe strain, with amino acid differences in the HN protein at position 335; a Glu in the wild-type and a Lys in the mutant. Again, the authors concluded that amino acid 335 was potentially responsible for adaptation to the cell culture system used in the study. No other genes were investigated in this study.

Structural changes and alterations in pathogenicity of a virulent virus have also been observed during serial passage *in vivo* (Love *et al.*, 1986). The Kilham strain of MuV was passaged in the brains of newborn hamsters, either with an interval of 4-5 days between inoculation and harvest or with an interval of 10-12 days. This difference in the mode of passaging resulted in the selection of variants which differed in their pathogenicity. Changes were observed, using monoclonal antibodies, in the NP protein and slight changes were also observed in the HN and P proteins. Differences in the fusion capability of the two variants as well as different tropisms for different cell types were observed.

B95a cells are a marmoset lymphoid cell line, transformed with EBV and have previously been shown to support the growth of other paramyxoviruses related to MuV, such as MV (Kobune *et al.*, 1990). It was observed that MV was subjected to host cell-mediated selection and that MVs grown in B95a cells were more representative of MV circulating among humans than were the viruses selected in Vero cells. Morbillivirus strains, RPV and CDV, have also been grown on B95a cells (Kobune *et al.*, 1991; Kai et al., 1993), where similar observations were noted.

Selection of variants by the host cell has also been observed for influenza virus (Robertson., 1993). Influenza virus grown in MDCK cells was shown to resemble the original wild type virus, whereas virus growth in hen's egg selected a virus with less resemblance to the wild type virus. Egg-adapted virus can be passaged in MDCK cells with no variant selection but MDCK-adapted virus passaged in the hen's egg selected a variant, suggesting that passage in the hen's egg was more restrictive than passage in cells. Substitutions were found in the egg-adapted virus located around the RBS in the HA protein, suggesting the involvement of the RBS in variant selection. Antigenic differences and differences in immunogenicity were also observed on occasion between the two types of variants. A second study by Robertson *et al* (1995) indicated that when MDCK-adapted virus was spiked with egg-adapted virus, the egg-adapted virus showed superior growth. The egg-adapted virus was shown to bind to and be internalised by MDCK cells with higher efficiency that the cell-adapted virus. The authors concluded that although the MDCK-adapted virus is indistinguishable from the wild type it is not the best fit for the MDCK receptor. The results of these studies by Robertson *et al* (1993 & 1995) can be related to a study by Ito *et al* (1997). Ito *et al* (1997) showed that a lack of SAα2,6Gal linkages in the allantois of chicken eggs is a selective pressure for the appearance of viral host cell variants with altered receptor specificity. The authors showed that viruses maintained high SAα2,6Gal specificity when grown in MDCK cells but passage in the allantois or amnion resulted in a complete shift to SAα2,3Gal specificity.

1.12 THE AIMS OF THIS THESIS

Prior to the commencement of this project only two cell substrates (Vero and CEF cells) were in use at NIBSC for studies relating to MuV infection *in vitro*. The suitability of Vero cells for the isolation and propagation of clinical samples and the assaying of mumps vaccines is under question after a previous studies have concluded that growth of MuV in Vero cells produces virus populations that are different from the original isolate (Yates, 1995 & Yates *et al.*, 1996). The major aim of this project was to give an understanding to the mechanisms involved in host cell passage of MuV and to move towards an optimum cell substrate with which to study MuV infection *in vitro*. The aim was achieved by the application of tissue culture and molecular biology techniques including:

- Analysis of virus infectivity after growth in different cell substrates using the $TCID_{50}$, HA and NA assays.
- Analysis of plaque morphology and antigenicity of viruses after growth in different cell lines.
- Molecular analysis of the nucleotide and amino acid sequences of the two envelope glycoproteins.

This project was undertaken to determine if an optimum host cell line, more appropriate for growth of MuVs, is available. The significance of growing MuVs in an optimum cell line is that analysis of *in vitro* MuV infection would more closely resemble infection of wild-type MuV.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CELL CULTURE MEDIA AND SOLUTIONS

RPMI 1640;	SIGMA pH 7.0
MEM;	GIBCO 0.97g MEM in 100ml GDW pH 7.0
PENSTREP;	1.25g penicillin-G sodium salt 1g streptomycin sulphate in a final volume of 100ml MEM
FUNGIZONE;	0.02g fungizone in a final volume of 100ml GDW
BICARBONATE STOCK;	4.4g $NaHCO_3$ 0.5g phenol red in a final volume of 100ml GDW
L-GLUTAMINE STOCK;	2.9g glutamine in a final volume of 100ml GDW
FCS	SIGMA
GROWTH MEDIUM;	3% (v/v) Bicarbonate stock 1% (v/v) penstrep 1% (v/v) fungizone 4% (v/v) FCS in MEM pH 7.0
MAINTENANCE MEDIUM;	3% (v/v) Bicarbonate stock 1% (v/v) penstrep 1% (v/v) fungizone 2% (v/v) FCS in MEM pH 7.0
SALINE 'A';	0.8g NaCl 0.04g KCl 0.1g glucose in a final volume of 100ml GDW
PBS;	1.07g NaCl 0.03g KCl 0.017g $CaCl_2.2H_20$ 0.013g $MgCl_2.6H_2O$ 0.40g $Na_2HPO_4.12H_2O$ 0.03g KH_2PO_4 in a final volume of 100ml GDW pH 7.4
PBS 'A';	1g NaCl 0.025g KCl 0.144g Na_2HPO_4 anhydrous

	0.025g KH_2PO_4 in a final volume of 100ml GDW pH 7.4
CEF WASH BUFFER;	1ml penstrep 1ml fungizone in 100ml PBS'A'
TRYPSIN STOCK;	SIGMA 1000-1500 BAEE units/mg 6ml trypsin in 100ml saline 'A'
EDTA STOCK;	0.4g EDTA in a final volume of 100ml saline 'A'
TRYPSIN SOLUTION;	1ml trypsin stock 1ml EDTA stock 1ml Bicarbonate stock in 100ml saline 'A'
CEF TRYPSIN SOLUTION;	1ml trypsin solution in 100ml CEF wash buffer
CMC;	SIGMA
CMC for overlay;	3.2g CMC in 100ml PBS 'A'
BACTOAGAR;	1.4g bactoagar in 100ml dH_20
METHYL VIOLET STAIN;	2.5g methyl violet stain 100ml ethanol in a final volume of 500ml GDW

2.1.2 NEURAMINIDASE ASSAY REAGENTS

PERIODATE REAGENT;	42.8g sodium metaperiodate 62ml orthophosphoric acid in 100ml dH_2O
ARSENITE REAGENT;	10g sodium arsenite 7.1g sodium sulphite 0.3ml concentrated sulphuric acid in 100ml dH_20
THIOBARBITURIC ACID REAGENT;	7.1g sodium sulphate 0.6g thiobarbituric acid in 100ml dH_20
FETUIN	SIGMA

2.1.3 MOLECULAR BIOLOGY SOLUTIONS

TRIS;	1M Tris-HCL pH 7.5
SDS;	10% SDS in GDW
PROTEINASE K COCKTAIL;	0.1M Tris pH 8.4 0.02M EDTA stock

	0.4M NaCl 40% (v/v) SDS stock solution 10% (v/v) proteinase K (20mg/ml) 0.4% glycogen (20mg/ml) in a final volume of 1ml GDW
PHENOL SOLUTION;	AquaPhenol, Appligene equal amount chloroform added
10xTBE;	54.5g Tris-HCL (pH 7.5) 27.85g 7.5M boric acid 4.65g 10mM EDTA in a final volume of 500ml GDW
2mM dNTPs;	Boehringer Mannheim Reagents 10mM dATP 10mM dCTP 10mM dGTP 10mM dTTP in dH_2O
1.25mM dNTPs;	Boehringer Mannheim Reagents 100mM dATP 100mM dCTP 100mM dGTP 100mM dTTP in dH_2O
ddATP;	Boehringer Mannheim Reagents 350mM ddATP 10mM dATP 10mM dCTP, dGTP, dTTP in dH_2O
ddCTP;	Boehringer Mannheim Reagents 16mM ddCTP 10mM dCTP 10mM dATP, dGTP, dTTP in dH_2O
ddGTP;	Boehringer Mannheim Reagents 50mM ddGTP 10mM dGTP 10mM dATP, dCTP, dTTP in dH_2O
ddTTP;	Boehringer Mannheim Reagents 600mM ddTTP 10mM dTTP 10mM dATP, dCTP, dGTP in dH_2O
PHIGEL 1;	Fisher Scientific acrylamide/bisacrylamide (19:1)
SEQUENCING GEL MIXTURE; (For one gel (50ml))	21g urea 7.5ml Phigel 1 22.5ml H_2O

	5ml 10xTBE buffer
BPB DYE;	0.025g BPB 0.025g xylene cyanol in 1ml 10xTBE 3ml glycerol 6ml dH_2O
FORMAMIDE DYE;	0.8ml 0.5M EDTA 0.1% (w/v) xylene cyanol 0.1% (w/v) BPB in formamide (saturated with Amberlite)
DTT;	0.1M DTT Prepared in 20ml 0.01M sodium acetate, pH 5.2
PREP-A-GENE BINDING BUFFER;	6M sodium perchlorate 50mM Tris-HCL pH 7.5 10mM EDTA in GDW
PREP-A-GENE WASH BUFFER;	20mM Tris-HCL pH 7.5 2mM EDTA 0.4M sodium chloride in GDW In an equal volume of ethanol
WACKER SOLUTION;	2.5ml ethanol 75µl, 10% glacial acetic acid 7.5µl, methmethylacryloxypropyl -trimethyloxysilane
NUCLEICLEAN WASH BUFFER;	Sigma 3ml wash buffer from kit 6.75ml GDW 10.25ml ethanol

2.1.4 OLIGONUCLEOTIDE PRIMERS

HN GENE PRIMERS (positions are expressed in relation to the HN gene, a diagram of the genome is presented in section 1.2.3):

SH-7	mRNA sense, at position -109 to -88 GAT CAA TCA CTC TAG AAA GAT C
HN-6	mRNA sense, at position 258 to 276 GAT CAA CTT GAG CAA TCA G
HN-18	mRNA sense, at position 572 to 592 GCC TAG CTT TAT CCA ACT GC
HN-19	reverse sense, at position 591 to 574 GCA GTT GGG ATA AAG CTA GGC
HN-2	reverse sense, at position 1059 to 1038 CCT GCA TAT GGG GGT GTC TTG C
HN-3	mRNA sense, at position 888 to 907

TCC ACC CCA CCT ACC GAA AC

HN-5 reverse sense, at position 1272 to 1252
GCG AGC TAG TTG TCC CCT CAA

HN-7 mRNA sense, at position 1148 to 1168
AGA TCA GCG TGC TTT GAG ATC

HN-8 mRNA sense, at position 1416 to 1435
GTG AAT ATG TCC TGG ATA CC

HN-9 reverse sense, at position 1725 to 1706
CAC CTG CTT TCA AGA TAC CG

L-19 reverse sense, at position + 111 to + 92
CAT TTG GTA ACT GGC CAT GC

F GENE PRIMERS (positions are expressed in relation to the F gene, a diagram of the genome is presented in section 1.2.3):

M-4 mRNA sense, at position -430 to - 409
ACC AAC CAT TCT AGT CCA TGC

F-9 reverse sense, at position 186 to 165
GTC AGG CAA CTA AGC TAT TAC

F-3 reverse sense, at position 448 to 430
GCC GCT GTC TCA TTC GTT CA

F-4 reverse sense, at position 748 to 729
GCC TTG AGG TCT TTG CTT GG

F-10 mRNA sense, at position 553 to 574
GCG GTA CAA GCA ATA CAA GAC C

F-16 reverse sense, at position 996 to 978
GCC AGA CAG GAT CTT GGA G

F-12 reverse sense, at position 1170 to 1151
GGA GTT ATA TGA GGC GAT T

F-6 mRNA sense, at position 1083 to 1105
GCC AAT ACA ATG AGG CAG AGA G

F-14 reverse sense, at position 1334 to 1317
GGA TTT CAG CAT TGT CTC

F-2 reverse sense, at position +49 to + 27
AAG AAT GAA TCT CCT AGG GTC G

2.2 METHODS

2.2.1 CELL LINES

Six cell lines were used in this project; B95a, BCL, CEF, HeLa, MRC-5 and Vero.

B95a cells

Transformed monkey B-lymphocytes (B95a), were a gift from Dr. K. Katrack at NIBSC. The cells secrete a high titre of Epstein Barr virus (EBV), providing a source of EBV to establish continuous transformed cells. B95a cells were grown in $25cm^2$ tissue culture flasks, in RPMI 1640 (SIGMA) containing 1% L-glutamine, 1% penstrep and 8% foetal calf serum (FCS) at 35°C, 5% CO_2. The cells were subcultured every four days by gentle resuspension of the cell monolayer to remove clumps of cells and the content of each flask split between three new $25cm^2$ tissue culture flasks (a split ratio of 1:3) and 10ml fresh medium added.

BCL cells

Mouse B cell leukaemia cells (BCL), were a gift from Dr. A. Meager at NIBSC. BCL cells were grown in suspension in $25cm^2$ tissue culture flasks with RPMI 1640 containing 10% FCS, 1% penstrep and 1% fungizone at 35°C, 5%CO_2. The cells were subcultured every four days by gentle resuspension of the cell suspension to remove clumps of cells and subcultured at a ratio of 1:4.

CEF cells

Chick embryo fibroblast cells (CEF) were prepared as required. A hen egg was swabbed with 70% IMS, the embryo removed using scissors and forceps and placed in a petri-dish containing wash buffer. The head, limbs and gut were removed and the embryo body rinsed twice in clean CEF wash buffer. The embryo body was cut up finely with scissors, placed into a conical flask containing 15ml CEF trypsin solution and stirred at 37°C for 30 minutes. The resulting cell suspension was filtered through Whatman filter paper, grade 1

and the cells in the filtrate pelleted by centrifugation at 1,000 rpm for 10 minutes. The supernatant fluid was discarded, the cells resuspended in growth medium containing 5% FCS, dispensed into two 25cm^2 tissue culture flasks and incubated at 35°C, 5% CO_2 until confluent.

HeLa cells

Human carcinoma epithelial cells (HeLa) were derived originally from a cervical carcinoma of a 31 year old woman and were available from stock at NIBSC. HeLa cells were grown routinely in 75cm^2 tissue culture flasks in growth medium containing 5% FCS at 35°C. The cells were subcultured every week by trypsinisation; the cell monolayer was washed twice with trypsin solution and incubated with a small amount of the final wash until the cells lifted off from the tissue culture flask. The cell suspension was finally aspirated with 10ml growth medium and subcultured at a ratio of 1:10.

MRC-5 cells (ATCC number CCL-171)

Human foetal lung cells (MRC-5) (Jacobs *et al.*, 1970) were originally derived from the lung of a normal human male foetus and were held in stock at NIBSC. MRC-5 cells were grown routinely in 75cm^2 tissue culture flasks in growth medium at 35°C and were subcultured each week by trypsinisation and gently resuspending the cell suspension with 8ml growth medium containing 5% FCS. The cells were subcultured at a ratio of 1:4.

Vero cells (ATCC number CCL-81)

African Green Monkey kidney cells (Vero) (Yasmura and Kawakita, 1963) were derived originally from the kidney of a normal African Green monkey. The cells were originally obtained from Merck Sharpe and Dohme (MSD). Vero cells were grown routinely in 16oz glass tissue culture bottles in growth medium at 35°C and were subcultured every three to four days by trypsinisation and gently resuspending the cell suspension with 6ml of growth medium. The cells were subcultured at a ratio of 1:6.

2.2.2 VIRUS STOCKS

Seven mumps strains were used in this project, JL-2, JL-5, Ur PT1, Ur PT3, Enders, Rubini and Po15/t. The two components of the Jeryl Lynn bulk vaccine, JL-2 and JL-5, were plaque purified three times in Vero cells from the original vaccine (the original JL vaccine was obtained from MSD). The two components of the Urabe bulk vaccine, 035 1.2.1 (Ur PT1) and 035 2.2.1 (Ur PT3) were also plaque purified three times in Vero cells from the original vaccine (the original Urabe vaccine was obtained from SmithKline Beecham (SKB)). Plaque purification involved performing a plaque assay (section 2.2.7), picking an isolated plaque and resuspending the plaque in 0.1ml maintenance medium. This resuspended plaque was passaged once, a plaque assay performed on the harvested material and the cycle repeated.

The Enders strain was a gift from B. K. Rima, Queens University, Belfast and the stock held at NIBSC was obtained by passaging this virus once on Vero cells. The Rubini strain was obtained from the Swiss Serum Institute and the stock held at NIBSC was obtained by passaging this virus four times on Vero cells. The Po15/t strain was isolated in Vero cells from a throat swab of a patient showing clinical symptoms of mumps in Portugal in 1996, and this isolated virus was passaged a further two times in Vero cells to produce the stock held at NIBSC. All viruses were stored at -70°C at NIBSC.

The working stocks of JL-2, JL-5, Ur PT1, Ur PT3 and Po15/t used in this study were obtained from the stock held at NIBSC. The Enders and Rubini strains used in this study were obtained from the stocks held at NIBSC but were passaged one further time in Vero cells to produce a working stock.

2.2.3 VIRUS PROPAGATION

Viruses were propagated using the following technique:

Growth medium was aspirated from a $25cm^2$ tissue culture flask containing an 80% confluent cell monolayer of the appropriate cell type (approximately $5x10^6$ cells) and the appropriate dilution of virus inoculum, diluted in maintenance medium, added. The appropriate dilution of virus inoculum was initially 3 $\log_{10}$ $TCID_{50}$/ml of virus, giving an approximate multiplicity of infection (MOI) of 0.0002 (1 $TCID_{50}$ per 5000 cells). The cells were incubated with virus for approximately 2 hours in a humidified incubator at 35°C, 5% CO_2, after which the inoculum was aspirated and fresh maintenance medium added. The infected cells were incubated at 35°C, 5%CO_2 until 80% CPE was observed. If no CPE was observed the infected cells were harvested 10 days post infection. Virus was propagated in BCL cells by adding virus inoculum into the freshly subcultured cell suspension and the cells incubated without the removal of the inoculum.

Virus was harvested by freezing the tissue culture flask containing infected cells at -70°C and thawing at room temperature to release any cell bound virus. The cell debris was then removed from the virus by centrifugation at 160 x g for 10 minutes. The supernatant fluid, containing virus, was aliquoted and stored at -70°C.

2.2.4 VIRUS TISSUE CULTURE INFECTIVITY DOSE ASSAY ($TCID_{50}$)

The infectivity of the viruses was determined using the $TCID_{50}$ assay. Virus was diluted 1/3 in maintenance medium and 150µl of this dilution dispensed into each of eight wells in the first column of a 96 well tissue culture plate. One hundred microlitres of maintenance medium were added to all other wells. Fifty microlitres of virus were sequentially transferred across the tissue culture plate resulting in a 1 in 3 dilution series of virus. One hundred microlitres of Vero cells were added to each well (approximately $1\text{-}5x10^4$ cells/well) and the plate incubated at 35°C for 7 days. The microscopic observation of

CPE indicated a positive result. The titre of the virus was the dilution producing CPE in 50% of tissue culture wells and was calculated using this simplified form of the Spearman-Karber formula:

$$m = X_K + d/2 - d\Sigma p_i$$

where:

m = the logarithm of the titre, relative to the test volume.

X_K = the logarithm of the smallest dose which induces infection of all cultures.

d = the logarithm of the dilution factor

p_I = the observed reaction rate

2.2.5 HAEMAGGLUTINATION ASSAY

Infectious tissue culture fluid was diluted in PBS 'A' in a doubling dilution series, from neat, across a 96 well V bottomed tissue culture plate in 50µl amounts. Fifty microlitres of 0.7 % (v/v in PBS) red blood cells (turkey, monkey, sheep or horse) were added to each well and the plate incubated at 4°C for 1 hour. The HA titre in HA units (HAU) of the virus was the reciprocal of the highest dilution observed to give complete agglutination of red blood cells (Fazekas de St Groth and Webster, 1966).

2.2.6 NEURAMINIDASE ASSAY

The neuraminidase assay used in this study was a colorimetric assay using fetuin as a substrate. The assay is based on measurement of the amount of sialic acid released from fetuin.

Virus was titrated in a doubling dilution series from neat tissue culture fluid to 1/128 in dH_2O. Fifty microlitres of each dilution were transferred to separate test tubes and 50µl fetuin (50mg/ml) added. The samples were incubated at 37°C for 18 hours, allowed to

cool to room temperature and 100µl periodate reagent added to each sample, after which the samples were incubated at room temperature for 20 minutes. One millilitre of arsenite reagent was added to each sample and vortexed until the colour disappeared. Two and a half millilitres of thiobarbituric acid reagent were added to each sample, the samples mixed and placed in a boiling water bath for 15 minutes. The titre of the viral NA was taken as the reciprocal of the highest dilution of virus to show a pink colour and was expressed in NA units (NAU).

2.2.7 ASSESSING PLAQUE MORPHOLOGY

Appropriate dilutions of virus were prepared in maintenance medium. One hundred and fifty microlitres of each dilution were dispensed into separate wells of a six well tissue culture plate and 3ml Vero cell suspension added per well ($1x10^6$ cells/well). The plate was incubated in a humidified incubator at 35°C, 5% CO_2 for 3 to 4 hours until the cells formed a confluent cell monolayer. The medium, containing virus inoculum, was aspirated and 3ml CMC. CMC overlay was prepared by adding 100ml 1xMEM to 50ml CMC and mixing. Eight ml FCS, 2ml fungizone, 2ml penstrep and 6ml sodium bicarbonate were then added. The tissue culture plate was incubated at 35°C, 5%CO_2 for 7 days.

After 7 days the overlay was removed, the cell sheet washed in PBS and stained with methyl violet solution for thirty minutes. The unbound stain was removed with water. The titre of the virus, expressed as pfu/ml, was determined by counting the observed plaques and taking into account inoculum volume and dilutions made.

2.2.8 MONOCLONAL ANTIBODIES

The monoclonal antibodies used in this project were either held in stock at NIBSC at -20°C as ascites fluid or were stored in liquid nitrogen as hybridoma cell lines that were grown as and when needed.

Hybridoma cells

Hybridoma cell lines were reconstituted for use by washing the cells twice in RPMI 1640 medium containing 20% FCS, at 160 x g. The pellet of cells was then resuspended in 10ml growth medium containing 10% FCS, dispensed into a 25cm^2 tissue culture flask and incubated at 35°C until confluent. The cells were resuspended by gentle aspiration, dispensed into a 75cm^2 tissue culture flask and incubated at 35°C until confluent. The cells were resuspended by gentle aspiration and split between two 75cm^2 tissue culture flasks. This process was repeated until six confluent 75cm^2 tissue culture flasks were obtained.

Production of tissue culture fluid containing monoclonal antibody

Hybridoma cell tissue culture fluid usually contained microgram quantities of monoclonal antibody; this was increased to milligram quantities by propagating the hybridoma cells in a mini-perm fermenter (Heraeus Instruments). The hybridoma cells of six confluent 75cm^2 tissue culture flasks were pelleted by centrifugation at 160 x g for 7 minutes. The pellets were pooled and resuspended in 37ml growth medium containing 20% FCS. Two millilitres of the cell suspension was used to re-seed a 25cm^2 tissue culture flask. A mini-perm fermenter was assembled according to the manufacturer's instructions and 400ml of growth medium, containing 10% FCS, decanted into the top compartment. Thirty-five millilitres of cell suspension was injected into the cell unit using a syringe to deliver the cells through a small porthole. The mini-perm fermenter was placed on rollers in an incubator at 35°C. The growth medium was replaced every 2 to 3 days.

After 3 weeks, 15ml cell suspension was removed from the cell unit and the cells pelleted by centrifugation at 160 x g for 5 minutes. The supernatant fluid, containing monoclonal antibody, was stored at -20°C for use and the pellet was resuspended in growth medium containing 20% FCS and replaced in the mini-perm fermenter. The dilution of tissue culture fluid containing monoclonal antibody to use in virus antigenic assays was then determined.

Ascites fluid

Ascites fluid containing monoclonal antibody had been produced previously (Yates *et al.*, 1996) and was held at -20°C. The following method had been used to produce the ascites fluid: hybridoma cells from six 75cm^2 confluent tissue culture flasks were pelleted by centrifugation and the supernatant fluid discarded. The pellets were resuspended and pooled in serum-free growth medium. The resuspended pellets were centrifuged for 5 minutes at 160 x g and the supernatant fluid discarded. The pellet of cells was resuspended in 3ml serum-free growth medium. Half a millilitre of the cell suspension was then injected into each of six mice previously inoculated intraperitoneally with 0.5ml of pristane oil, to condition the abdomen. After 1 week the mice had produced ascites fluid and were sacrificed. The ascites fluid was collected and placed at 4°C overnight to clot. The top layer of fluid was removed and centrifuged at 880 x g for 5 minutes. The supernatant fluid was removed and stored at -20°C ready for use.

2.2.9 VIRUS NEUTRALISATION ASSAYS

Virus was diluted in maintenance medium containing 1/100 monoclonal antibody dilution (MAb neutralisation titres had been determined previously by Yates *et al.*, (1996) and are presented in Table 2.1) to give 500TCID$_{50}$/ml or, where virus was at a lower titre than this, virus was used undiluted. Respective positive control virus was also included with no addition of MAb. The sample was then incubated at 4°C for 90 minutes. Fifty microlitres of sample were added to triplicate wells on a 24 well tissue culture plate and 1ml Vero cell suspension at 1-5x10^5 cells/ml containing a 1/100 dilution of monoclonal antibody added.

After incubation of the samples at 35°C for 3 hours the medium was aspirated and 1ml CMC overlay containing a 1/100 dilution of monoclonal antibody added. The plates were incubated at 35°C for 7 days, washed in PBS and stained with methyl violet and this stain

removed with water. The reactivity of seven different MAbs was analysed for each virus, MAbs 1637, 1641, 1648, 1689, 1721, 1726 and 1970.

Table 2.1: The biological activity of monoclonal antibodies against the parental (Urabe) strain of mumps (Yates *et al.*, 1996).

MAB	NEUTRALISATION TITRE	III TITRE	NI TITRE
1637	100 000	<20	500
1641	1 000	<20	500
1648	10 000	<20	1 000
1689	100 000	>2560	100
1721	100 000	5 000	>100
1726	10 000	1 280	>100
1970	100 000	>20 480	>100

2.2.10 RNA EXTRACTION

RNA was extracted from infectious tissue culture fluid using a modified method of Chomzynski and Sacchi (1987). Two hundred and fifty microlitres of infectious tissue culture fluid were incubated at 50-55°C with 250µl Proteinase K cocktail. To this mixture 500µl phenol solution was added, vortexed for 1 minute and centrifuged at 11,600 x g for 10 minutes. The tissue culture fluid was removed from the phenolic layer, placed in a clean tube and 400µl chloroform added to remove excess phenol. The sample was vortexed and centrifuged at 11,600 x g for 5 minutes. The tissue culture fluid was removed from the chloroform layer, placed in a clean tube and 750µl ethanol added. The sample was inverted to mix and the RNA precipitated at -20°C for at least 60 minutes.

2.2.12 cDNA SYNTHESIS

The precipitated RNA was centrifuged at 11,600 x g for 30 minutes at 4°C, washed with ice cold ethanol and pelleted at 11,600 x g for 5 minutes and the pellet freeze-dried under vacuum for 20 minutes. The freeze-dried RNA pellet was resuspended in 4µl 2-

mercaptoethanol (1/20 dilution of stock, BDH Biochemicals), 2µl dH_2O and 2µl random primer (random hexamer) (1/30 dilution of stock at 500µg/ml, Promega) and incubated at 65°C for 5 minutes. The solution was then incubated at room temperature for 5 minutes. To the solution, the following was added; 1.5µl 10xAMV reverse transcriptase buffer (NBL Gene Sciences), 1.8µl dH_2O, 2.0µl 2mM dNTPs, 1.0µl 0.1M DTT, and 0.7µl AMV RTase enzyme (NBL Gene Sciences (20units/µl)). The mixture was incubated at 42°C for 45 minutes, after which the mixture was heated to 90°C for 30 seconds to destroy enzyme activity. The RNA-cDNA mixture was stored at -20°C until used in a PCR reaction.

2.2.12 POLYMERASE CHAIN REACTION

Oligonucleotide primers

The primers used in PCR were either held in stock at NIBSC or were obtained from Gibco BRL. Primers obtained from the manufacturers were reconstituted in 500µl 10xTE for 2 minutes. The optical density of a 1/20 dilution of this stock in dH_20 was determined at 260nm and the readings used to calculate the concentration of primer necessary for use in the PCR reaction using the following formula (provided by Gibco BRL);

Concentration µg/ml = A260 x molecular weight of primer x dilution factor

The concentration was then converted to picomoles and the dilution required to give 3.2 picomoles calculated (PCR and sequencing reactions were performed with primer at 3.2 picomoles, a value recommended for primer use by the manufacturers of the ABI Prism 310 Genetic Analyser, Perkin Elmer).

PCR reaction

To 4µl of cDNA the following reagents were added; 10µl 10xPCR buffer (containing 15mM $MgCl_2$, Perkin Elmer), 16µl 1.25 mM dNTPs, 5µl mRNA sense primer, 5µl antisense primer, 59.5µl dH_2O and 0.5µl AmpliTaq DNA polymerase, a polymerase from

the bacterium *Thermus aquaticus*, Perkin Elmer (5units/µl). To prevent evaporation of the sample, 80µl of paraffin oil was added to the sample. The sample was then amplified for thirty cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 90 seconds using a Techne Cyclogene thermocycler.

Examination of PCR product

Eight microlitres of PCR product were mixed with 3µl BPB dye and loaded onto a 1% agarose gel, (Seakem), in 1xTBE containing 30µl ethidium bromide (10mg/ml) per 100ml of agarose. The samples were then electrophoresed at 60mA for 60 minutes. The DNA bands in the gel were then visualised using an UV transilluminator.

2.2.13 MANUAL DNA SEQUENCING

Purification of PCR product

The paraffin oil was removed from the PCR product and 80µl chloroform added to absorb any remaining oil, the samples were mixed and centrifuged for 2 to 3 minutes at 11,600 x g. The aqueous layer containing the PCR product was removed from the chloroform and transferred to a 1.5ml reaction tube containing 200µl binding buffer and 3µl glass milk (Prep-a-Gene kit, Promega). The sample was incubated at room temperature for 20 minutes to allow the DNA to bind to the glass milk. The glass milk binds DNA fragments greater then 200 bases in length. This sample was centrifuged at 11,600 x g for 2 minutes, the supernatant fluid removed and the pellet resuspended in 500µl wash buffer. This wash process was repeated twice more. The final pellet was resuspended in 16µl dH_2O and incubated at room temperature for 20 minutes to allow release of the DNA from the glass milk. The purified DNA was collected by centrifugation at 11,600 x g for 30 seconds and the supernatant fluid, containing purified DNA, transferred to a clean tube. The purified DNA was quantified by loading 1µl DNA with 3µl BPB dye on an agarose gel. After electrophoresis the DNA was quantified approximately by visual inspection (a visual band

was approximately 50-100ng). Approximately 100ng of purified DNA were then used in sequencing reactions.

DNA sequencing primers

Messenger-RNA sense and antisense primers were used for nucleotide analysis of the HN and F genes. The primers were prepared for use as described previously. A list of the primers, their positions in relation to the genes and their nucleotide sequences can be found in the Materials section.

Radiolabelling of oligonucleotide primers (primer kination) (Maxam and Gilbert, 1980)

Three microlitres of primer, 2µl dH_2O, 1µl 10x polynucleotide kinase (PNK) buffer (Pharmacia Biotech), 3µl γ-P^{32} ATP and 1µl T4PNK enzyme (Pharmacia Biotech at 1000 units/µl) were added to a reaction tube, mixed and incubated at 37°C for 12 minutes. The mixture was then incubated for 30 seconds at 90°C to inactivate the enzyme and used in sequencing reactions.

Sequencing reactions

To a reaction tube, 2.3µl of 10xSuper Taq PCR buffer (HT Biotechnology), 3.3µl dH_2O, 1.5µl of radiolabelled oligonucleotide primer and 0.1µl Super Taq enzyme (HT Biotechnology), were added and mixed. Seven microlitres of this mixture were added to a reaction tube containing 4.0µl of purified DNA and mixed well. Two microlitres of this mixture were then transferred to each of four 0.5ml reaction tubes containing one of the four ddNTPs mixtures (A, C, G or T) and mixed well. One drop of paraffin oil was added to the samples, to prevent evaporation and the sequencing reactions subjected to amplification for twelve cycles of 94°C for 60 seconds, 50°C for 30 seconds and 72°C for 60 seconds. Two microlitres of formamide dye were then added to each tube and the sequence reactions were stored at -20°C until used.

Polyacrylamide gel electrophoresis

Two glass plates (20cm x 38cm x 0.4cm) were cleaned thoroughly and wiped with ethanol. One of the plates was coated with wacker solution and the second plate was coated with Repelcote (BDH Silicone Products), a water repellent. The plates were taped together to ensure no leakage of gel, wacker and repelcote sides facing together and separated with spacer strips (approximately 0.5mm thick).

The sequence gel mix was prepared and immediately prior to use, 500µl 10% ammonium persulphate, BDH Chemicals and 14µl N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma) were added to the gel mix. The gel mix was poured carefully between the glass plates, a comb inserted and held in place with clips. After polymerisation, the tape was removed from the bottom of the plates, the gel placed in a gel tank with 1xTBE buffer, the comb slowly removed and the wells washed out with buffer.

Prior to sample loading the gel was pre-run for 30 minutes at a voltage of ≤ 1,000V. Sequencing reaction samples were incubated in a boiling water bath for two minutes and placed directly on ice. One microlitre of each sequence reaction sample (A, C, G and T) was then loaded into each of four consecutive wells and electrophoresis performed at 1,300V for varying lengths of time, depending on the length of product under analysis (approximately 2-4 hours). After electrophoresis the gel was fixed in 10% glacial acetic acid for 20 minutes, rinsed in H_2O for 10 minutes to remove traces of urea and dried. An X-ray film was placed on top of the gel and placed in an autoradiograph cassette until ready to be developed.

2.2.14 NUCLEOTIDE SEQUENCING, using the ABI PRISM 310 GENETIC ANALYSER

Purification of the PCR product

Thirty microlitres of PCR product were added to 5µl BPB dye and loaded onto a midi-gel prepared as follows; 1% agarose in 1xTBE with 30µl ethidium bromide per 100ml agarose. The gel was electrophoresed at 60V for 60 minutes. DNA bands were visualised using an UV transilluminator, excised from the gel and placed in 1.5ml reaction tubes.

The PCR product was cleaned using a Nucleiclean kit obtained from SIGMA. The Nucleiclean kit provides a fast and simple method for the isolation of DNA or RNA from agarose gels. Five hundred microlitres of salt buffer (Nucleiclean kit, SIGMA) were added to each sample and the gel left to dissolve at 35°C for 30 minutes. Five microlitres of glass beads (Nucleiclean kit, SIGMA) were then added, each sample was vortexed and incubated at room temperature for 5 minutes to allow DNA to bind to the glass beads. The samples were centrifuged at 11,600 x g for 10 seconds, the supernatant fluid removed and 500µl wash buffer (Nucleiclean kit, SIGMA) added. The pellet was resuspended and centrifuged at 11,600 x g for 10 seconds. The wash process was repeated a further two times. After the final wash, the glass beads were resuspended in 10µl dH_2O and incubated at room temperature for 5 minutes to allow release of the DNA from the glass beads. The sample was then centrifuged for 10 seconds at 11,600 x g and the resultant supernatant fluid containing DNA transferred to a clean tube and stored at -20°C until used for sequencing reactions.

Oligonucleotide primers

Oligonucleotide primers used were the same as described previously.

Sequencing reactions

Sequence reactions were carried out using the dRhodamine Big Dye terminator cycle sequencing kit, (PE Applied Biosystems). To a 0.5ml reaction tube, 50-100ng of purified PCR product, 1µl of a 3.2pmole oligonucleotide primer dilution and 8µl terminator ready reaction mix (PE Applied Biosystems) were added. The quantity was made up to 20µl with dH_2O, mixed well and overlaid with 40µl paraffin oil. The terminator ready reaction mix contained four dideoxynucleotide terminators each separately labelled with a 3'-fluorescent dye, instead of the radioactive labels. The reactions were then subjected to amplification with the following conditions; 96°C for 1 minute and then twenty-five cycles of 96°C for 10 seconds and 55°C for 3 minutes.

Purification of extension products

To remove excess dye terminators and salt, both of which lowered the quality of the extension products, the reaction mixtures were subjected to an ethanol/sodium acetate precipitation procedure. For this, the 20µl extension product was transferred to a 0.5ml reaction tube containing 200µl ethanol, 10µl 3M sodium acetate and 70µl dH_2O. This mixture was left on ice for 10 minutes to allow precipitation of the DNA. The sample was centrifuged for 20 minutes at 4°C at 11,600 x g and washed twice in ice cold 70% ethanol. After the second wash, the pellet was air dried and then resuspended in 20µl template suppression reagent (ABI Prism kit, Perkin Elmer). The samples were heated to 96°C for 10 minutes prior to analysis in the ABI Prism 310 Genetic Analyser.

The ABI Prism 310 Genetic Analyser

The ABI Prism 310 Genetic Analyser, obtained from Perkin Elmer, was prepared according to the manufacturer guidelines. The genetic analyser is an automated instrument system capable of determining nucleotide sequences. After the DNA fragments are labelled, the genetic analyser automatically introduces the samples into a polymer filled

capillary for electrophoresis. The dye-labelled DNA fragments electrophorese through the polymer and separate according to size. A laser illuminates the samples, the fluorescent dyes become excited and emit a light at a specific wavelength. The fluorescent signal is collected and separated by a spectrograph according to wavelength and at the end of the run the computer automatically analyses the collected data.

CHAPTER III

GROWTH OF MuV IN DIFFERENT CELL SUBSTRATES

3.1 INTRODUCTION

Propagation of MuVs in Vero or CEF cells can alter their phenotypic and genotypic characters (Yates., 1995, Afzal *et al.*, 1998). For example, after two passages of the Urabe strain of MuV in Vero cells, the lysine at position 335 in the HN gene was replaced by glutamic acid, whereas, after two passages in CEF cells, no selection occurred (Yates, 1995). The Jeryl Lynn (JL) virus is heterogeneous, being composed of two variants, JL-2 and JL-5 (Afzal *et al.*, 1993). After passage of the JL virus in Vero cells, JL-2 outgrew JL-5. In contrast, JL-5 outgrew JL-2 after passage in CEF cells (Afzal, unpublished data).

The aim of this chapter is to determine whether an alternative cell line to Vero cells is available and appropriate for studying MuVs *in vitro*. An appropriate, alternative cell line is one in which infectious virus progeny is produced and where no, or little, genetic or phenotypic variation from the parental virus occurs.

3.2 EXPERIMENTAL PROCEDURES

JL-2, JL-5, Ur PT1, Ur PT3, Enders, Rubini and Po15/t (prepared in Vero cells as described in Chapter II and summarised in Table 3.1) were passaged five times in five different cell lines; B95a, BCL, CEF, HeLa and MRC-5 (Chapter II).

Table 3.1: Passage histories of the Vero-grown parental viruses.

Virus	Passage History
JL-2	From CEF-derived JL vaccine plaque purified three times in Vero cells
JL-5	From CEF-derived JL vaccine plaque purified three times in Vero cells
Ur PT1	From CEF-derived Urabe vaccine plaque purified three times in Vero cells
Ur PT3	From CEF-derived Urabe vaccine plaque purified three times in Vero cells
Enders	Passaged twice in Vero cells
Rubini	From MRC-5-derived vaccine and passaged 5 times in Vero cells
Po15/t	Wild-type virus, isolated by three passages in Vero cells

The viruses were grown on the different cell lines at an approximate multiplicity of infection (MOI) of 0.0002 (1 virus particle per 5000 cells). After an adsorption period of

2-3 hours the inoculum was aspirated and replaced with fresh maintenance medium (Chapter II). BCL cells grow in suspension and hence, virus at a MOI of 0.0002, was added into a freshly sub-cultured cell suspension and not aspirated after the adsorption period. The virus progeny were harvested, as described in Chapter II, when either 80-90% CPE was observed or after 10 days, if no CPE was observed. The progeny virus was passaged a further four times using the same procedure.

Cytopathic effect observed during virus passage was recorded for type and for the time taken (in days) between inoculation and appearance of CPE. The $TCID_{50}$ assay, performed on Vero cells (Chapter II) was used to determine the infectivity titre of virus after each passage level, and the infectivity titre was expressed in $\log_{10}$ $TCID_{50}$/ml. Where a virus titre was <3 $\log_{10}$ $TCID_{50}$/ml, the inoculum volume used for further passage was increased, to attempt to increase production of infectious virus. The haemagglutination (HA) and neuraminidase (NA) activities of the viruses were analysed after each passage level and the ratios between $TCID_{50}$ and HA, $TCID_{50}$ and NA, and HA and NA were also calculated.

3.3 RESULTS

3.3.1 CYTOPATHIC EFFECT OF MuV on its HOST CELL

Cytopathic effect is defined as any observed histological affect caused to cells when infected by a virus.

When grown in B95a cells (Figure 3.1) all viruses caused CPE at each passage level. Cytopathic effect became visible on the fifth day during the first passage in B95a cells but appeared earlier after subsequent passages until CPE was observed after the third day for fifth-passage virus. Cytopathic effect caused by JL-2, JL-5, Enders, Rubini and Po15/t was observed as multiple cell fusion, resulting in the appearance of multinucleated cells, cell death and in the production of formations of cells called spindle cells (Figure 3.2).

Cytopathic effect caused by Ur PT1 and Ur PT3 was mainly in the form of cell death; some cell fusion was observed but spindle cells were not formed (Figure 3.3).

The various MuVs, during the first passage in HeLa cells, caused CPE in the form of cell death and minimal cell fusion. However, during further passage increased cell fusion was observed. In contrast to the other viruses, Ur PT1 and Ur PT3 caused less cell fusion. Cytopathic effect was initially observed 5 days post inoculation; after further passage CPE was observed 3 days post inoculation.

No CPE was observed in MuV-infected BCL, CEF or MRC-5 cells.

3.3.2 EFFECT of CELL PASSAGE on MuV INFECTIVITY TITRES

Infectivity titres were measured for the Vero-grown parental viruses. The $TCID_{50}$/ml of Ur PT1 and Ur PT3 were the highest at 6.16 log_{10} and 6.01 log_{10}, respectively (Table 3.2). The infectivity titre of Enders was intermediate at 5.11 log_{10} and the infectivity titres of JL-2, JL-5, Rubini and Po15/t were slightly lower at 4.6 log_{10}, 4.72 log_{10} and 4.36 log_{10} respectively. The infectivity of each virus after each passage in each cell line was determined and the results are presented in Table 3.2 and are summarised in Table 3.3. Assays were performed in duplicate and an average value taken as the $TCID_{50}$/ml. Significant changes in infectivity titre were taken to be where an increase or decrease of > 1 log_{10} in the value of $TCID_{50}$/ml was observed.

Figure 3.1: A confluent uninfected B95a cell monolayer. The stippling effect observed is an artefact.

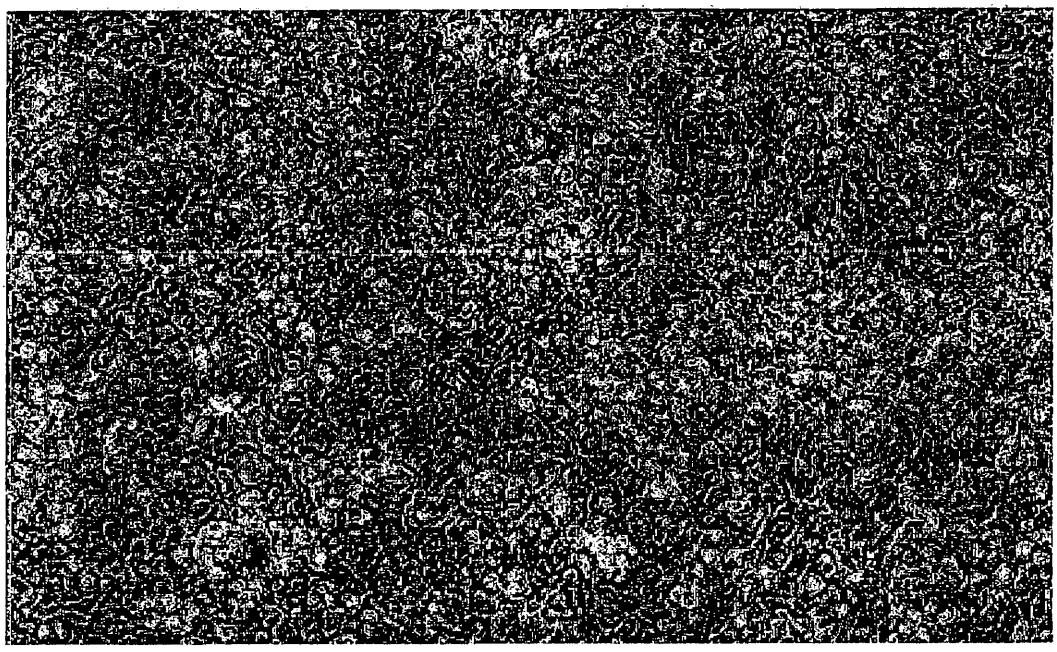


Figure 3.2: Cytopathic effect observed when B95a cells were infected with Enders; similar cytopathic effect was observed when B95a cells were infected with JL-2, JL-5, Rubini and Po15/t.

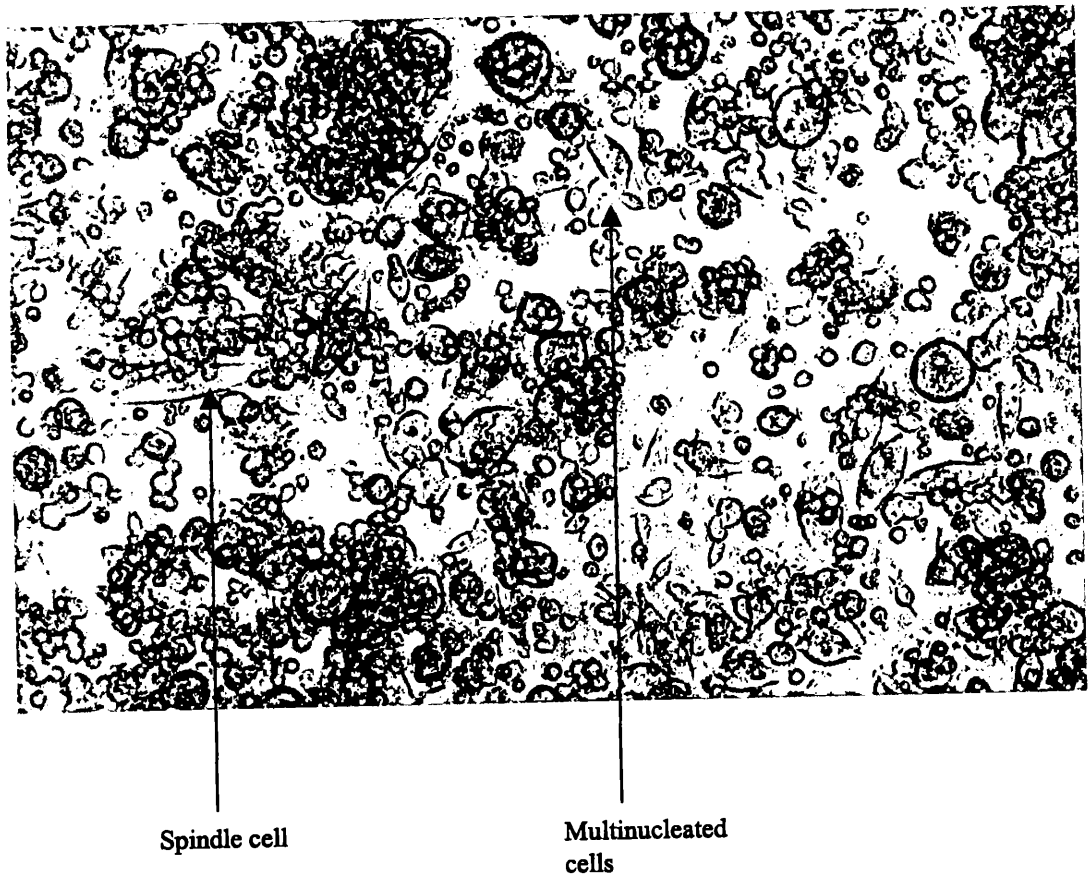


Figure 3.3: Cytopathic effect observed when B95a cells were infected with Ur PT1; a similar effect was observed when B95a cells were infected with Ur PT3.

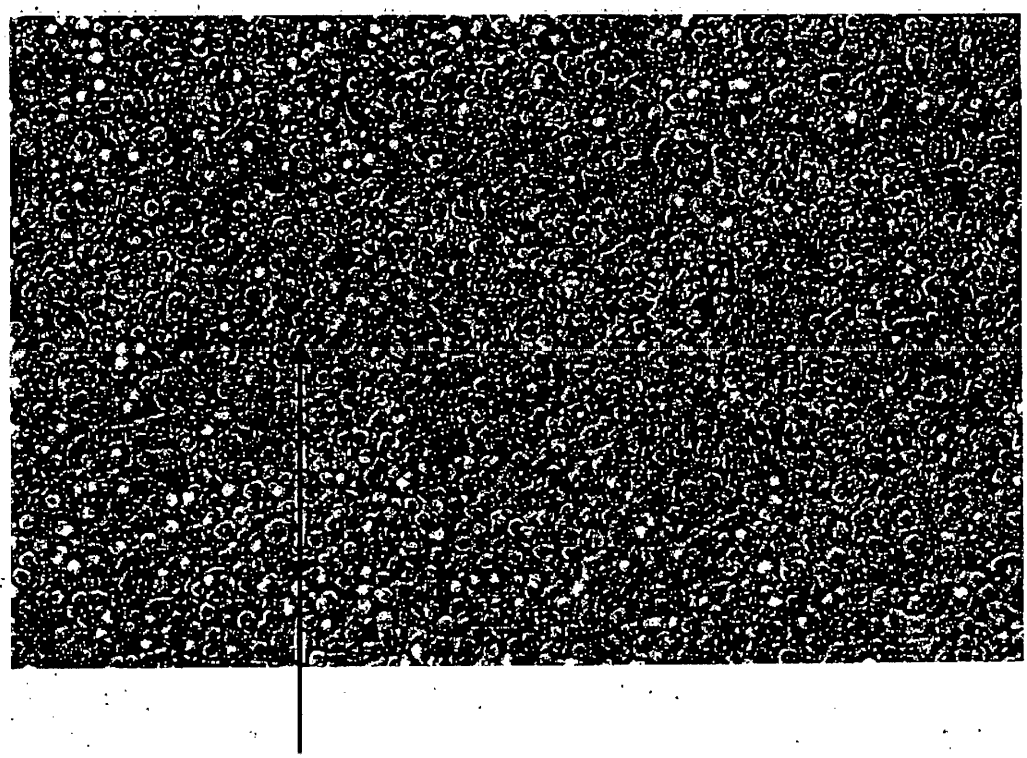


Table 3.2: Infectivity titres, expressed in log_{10} $TCID_{50}$/ml, of the parent and progeny viruses after passage in different cell lines.

VIRUS	LOG_{10} TITRE[1]	CELL LINE	LOG_{10} TITRE at PASSAGE LEVEL				
			P1	P2	P3	P4	P5
JL-2	4.60	B95a	4.42	4.96	7.06	6.94	5.86
		BCL	2.11	2.11	2.11	2.11	2.11
		CEF	3.22	2.11	2.11	2.11	2.11
		HeLa	2.38	5.32	5.14	5.86	5.44
		MRC-5	2.11	2.11	2.11	2.11	2.11
JL-5	4.72	B95a	2.11	2.11	5.26	6.10	6.94
		BCL	2.11	2.11	2.11	2.11	2.11
		CEF	2.18	2.11	2.11	2.11	2.11
		HeLa	2.44	2.80	4.90	6.28	6.22
		MRC-5	2.11	2.11	2.11	2.11	2.11
Ur PT1	6.16	B95a	5.62	6.28	6.10	6.82	7.42
		BCL	4.42	4.06	2.44	2.11	2.11
		CEF	2.38	5.80	2.11	2.11	2.11
		HeLa	5.14	5.92	5.50	6.28	7.24
		MRC-5	2.11	2.11	2.11	2.11	2.11
Ur PT3	6.01	B95a	6.64	6.34	5.44	5.58	7.06
		BCL	3.28	2.11	2.11	2.11	2.11
		CEF	3.28	2.11	2.11	2.11	2.11
		HeLa	4.90	5.74	6.70	7.36	6.88
		MRC-5	2.11	4.12	4.18	3.28	5.20
Enders	5.11	B95a	5.98	5.08	6.28	6.28	7.42
		BCL	2.11	2.11	2.11	2.11	2.11
		CEF	2.80	2.11	2.11	2.11	2.11
		HeLa	2.38	2.80	4.90	6.55	6.88
		MRC-5	2.11	2.11	2.11	2.11	2.11
Rubini	4.32	B95a	5.32	5.20	4.77	6.58	6.34
		BCL	2.74	2.11	2.11	2.11	2.11
		CEF	2.11	2.11	2.11	2.11	2.11
		HeLa	2.11	3.28	3.82	6.22	6.64
		MRC-5	2.11	4.00	2.11	2.11	2.11
Po15/t	4.36	B95a	5.20	4.96	7.00	7.18	6.58
		BCL	2.11	2.11	2.11	2.11	2.11
		CEF	2.11	2.11	2.11	2.11	2.11
		HeLa	2.92	3.22	6.88	6.58	5.20
		MRC-5	2.11	2.11	2.11	2.11	2.11

[1] Titre of Vero-grown parental virus

Table 3.3: Summary of the results of MuV passage in different cell lines.

CELL LINE	VIRUS	PASSAGE LEVEL				
		P1	P2	P3	P4	P5
B95a	JL-2	-[1]	-	↑[2]	↑	↑
	JL-5	↓[3]	↓	-	↑	↑
	Ur PT1	-	-	-	-	↑
	Ur PT3	-	-	-	-	↑
	ENDERS	-	-	↑	↑	↑
	RUBINI	-	-	-	↑	↑
	Po15/t	-	-	↑	↑	↑
BCL	JL-2	↓	↓	↓	↓	↓
	JL-5	↓	↓	↓	↓	↓
	Ur PT1	↓	↓	↓	↓	↓
	Ur PT3	↓	↓	↓	↓	↓
	ENDERS	↓	↓	↓	↓	↓
	RUBINI	↓	↓	↓	↓	↓
	Po15/t	↓	↓	↓	↓	↓
CEF	JL-2	↓	↓	↓	↓	↓
	JL-5	↓	↓	↓	↓	↓
	Ur PT1	↓	-	↓	↓	↓
	Ur PT3	↓	↓	↓	↓	↓
	ENDERS	↓	↓	↓	↓	↓
	RUBINI	↓	↓	↓	↓	↓
	Po15/t	↓	↓	↓	↓	↓
HeLa	JL-2	↓	-	-	↑	-
	JL-5	↓	↓	-	↑	↑
	Ur PT1	↓	-	-	-	↑
	Ur PT3	↓	-	-	↑	-
	ENDERS	↓	↓	-	↑	↑
	RUBINI	↓	↓	-	↑	↑
	Po15/t	↓	↓	↑	↑	-
MRC-5	JL-2	↓	↓	↓	↓	↓
	JL-5	↓	↓	↓	↓	↓
	Ur PT1	↓	↓	↓	↓	↓
	Ur PT3	↓	↓	↓	↓	-
	ENDERS	↓	↓	↓	↓	↓
	RUBINI	↓	-	↓	↓	↓
	Po15/t	↓	↓	↓	↓	↓

[1] No significant difference from the titre of Vero-grown parental virus
[2] Significant increase from the titre of the Vero-grown parental virus
[3] Significant decrease from the titre of the Vero-grown parental virus

B95a-derived MuVs

After five passages in B95a cells, all the MuVs had increased infectivity in comparison to their Vero-grown parental viruses (Table 3.2 and 3.3 and Figure 3.1).

Growth of the 7 MuVs in B95a cells appeared to stimulate the production of actively replicating progeny with significantly higher infectivity titres (by 1-2 $\log_{10}$ $TCID_{50}$/ml), than their respective parent virus (Tables 3.2 and 3.3). No apparent adaptation period was observed, with the possible exception of the JL-5 virus. The infectivity of this JL-5 virus dropped, by a significant value, for the initial two passages and then increased, suggesting adaptation to the cell line on, or after, the third passage. All other viruses increased in infectivity over increasing passage without an initial period of decreased infectivity.

HeLa-derived MuVs

In addition to B95a cells, growth in HeLa cells also appeared to support active replication of the 7 MuVs, with significantly higher infectivity titres for 4 of the MuVs when compared to the Vero grown parental, after an initial lag period of decreased infectivity (Tables 3.2 and 3.3 and Figure 3.2). The initial decreased infectivity, followed by an increase after the third passage, suggests the occurrence of adaptation to the cell line by the viruses. After the fourth passage, six of the MuVs had significantly higher infectivity titres than their respective parental virus, this value then appeared to plateau, with only four of these viruses having significantly higher infectivity titres after the fifth passage.

Growth of MuV in BCL, CEF or MRC-5 cells

After five passages in BCL and CEF cells, all the MuVs had decreased infectivity in comparison to their Vero-grown parental viruses (Tables 3.2 and 3.3). After five passages in MRC-5 cells, all the MuVs, bar one (Ur PT3), had decreased infectivity in comparison to their Vero-grown parental viruses; Ur PT3 had a similar level of infectivity to its Vero-grown parent (Table 3.2). The titres observed probably arise from remaining input virus

Figure 3.4: Growth of MuV in B95a cells

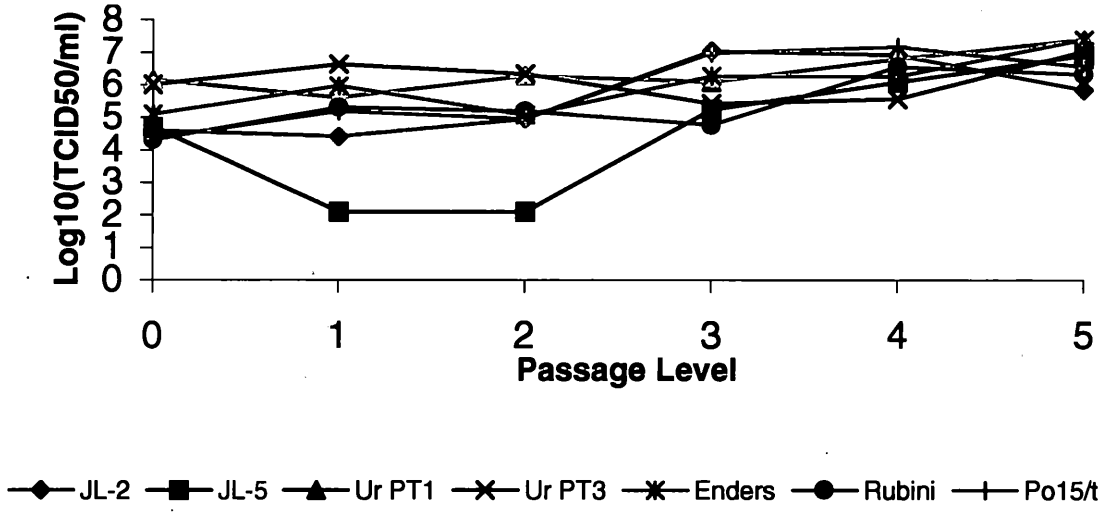
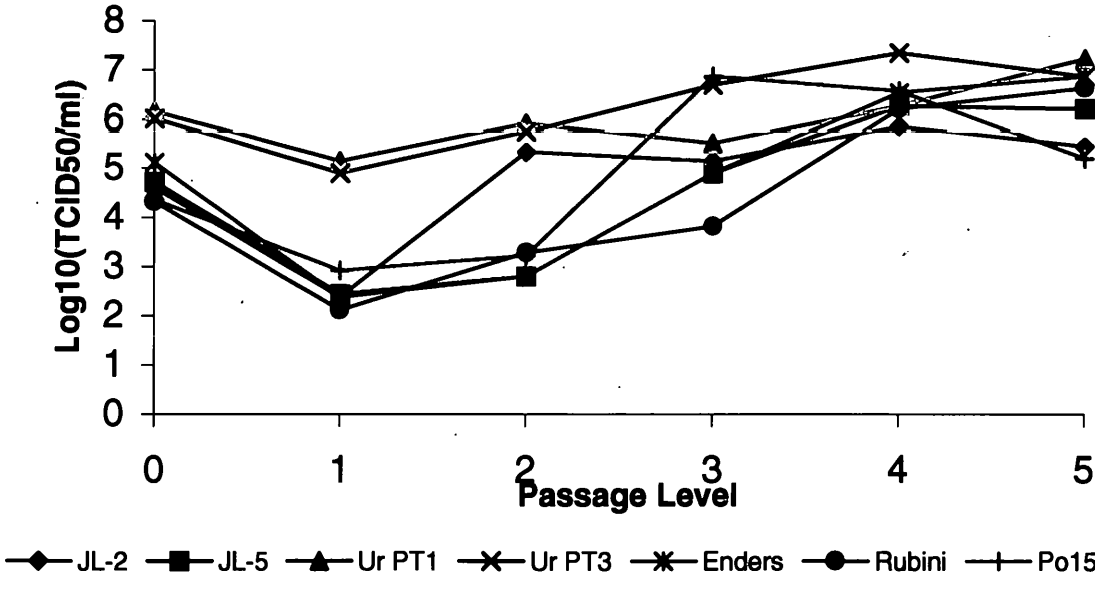


Figure 3.5: Growth of MuV in HeLa cells



(with the exception of Ur PT3 grown in MRC-5 cells) and it is likely that no productive replication was taking place.

3.3.3 EFFECT of CELL PASSAGE on MuV HA TITRES

Haemagglutination assays were performed as described in Chapter II at 4°C to avoid interference of haemagglutination by neuraminidase activity and were performed on all Vero-grown viruses and their progeny from each passage level. Neat tissue culture supernatant from infected cells was assayed and four species of erythrocyte, turkey, sheep, horse and monkey, were employed. Turkey erythrocytes are routinely used for assessing the HA titre of MuV and influenza viruses. Sheep, horse and monkey erythrocytes were included in this assay to determine if any significant differences in HA occur with the use of these erythrocytes, in comparison to the turkey erythrocytes.

Results of the HA assays performed on the parental viruses are presented in Table 3.4 (A and B). No HA activity was detected for the JL-2 parental virus, whilst JL-5 had an average HA titre of 8 HAU. The HA of Ur PT1 ranged from 2 HAU with horse RBCs to 16 HAU with sheep RBCs, whilst the HA titre of Ur PT3 was several fold higher at 64 HAU. Enders had an HA titre of between 16 to 32 HAU, whilst Rubini had a higher HA titre of 64 HAU. Po15/t had a range of HA titres from undetectable with horse RBCs to 16 HAU with monkey and sheep RBCs. These results suggested that HA assays performed with horse erythrocytes may give poor results for Ur PT1 and Po15/t progeny viruses and that JL-2 progeny viruses could also give poor results with all four RBC types. Significant changes in HA activity were taken where a > 4-fold increase or decrease was observed between parent and progeny viruses.

Table 3.4: Results of the HA assays performed on virus progeny. HA assays were performed at 4°C, using four species of erythrocyte.

A) HA assay results for MuV grown in B95a cells.

VIRUS	RBC[1]	P0[2]	P1	P2	P3	P4	P5
JL-2	Turkey	-[3]	64	-	4	32	4
	Sheep	-	64	2	16	32	8
	Horse	-	64	-	-	32	2
	Monkey	-	64	1[4]	16	16	4
JL-5	Turkey	8	-	-	-	16	32
	Sheep	16	-	-	32	64	64
	Horse	4	-	-	-	32	64
	Monkey	8	-	-	-	16	32
Ur PT1	Turkey	4	-	16	8	8	32
	Sheep	16	-	16	16	16	32
	Horse	2	-	1	-	-	-
	Monkey	8	2	2	16	16	32
Ur PT3	Turkey	64	64	64	64	64	64
	Sheep	64	64	128	128	128	64
	Horse	64	8	8	8	16	16
	Monkey	64	64	128	256	128	64
Enders	Turkey	16	32	32	64	16	64
	Sheep	32	16	32	128	16	64
	Horse	16	16	16	64	8	64
	Monkey	16	32	32	64	16	64
Rubini	Turkey	64	32	64	32	16	16
	Sheep	64	32	64	32	16	16
	Horse	64	32	64	16	16	16
	Monkey	64	64	64	16	16	16
Po15/t	Turkey	4	16	8	8	32	4
	Sheep	16	32	16	16	64	8
	Horse	-	-	-	-	-	-
	Monkey	16	32	16	16	64	16

[1] RBC = Red blood cell
[2] P0 = Vero-grown parental virus
[3] - = Negative
[4] 1 = Neat only was positive for haemagglutination

Table 3.4 continued:

B) HA assay results for MuV grown in HeLa cells.

VIRUS	RBC[1]	P0[2]	P1	P2	P3	P4	P5
JL-2	Turkey	-[3]	-	8	32	16	32
	Sheep	-	-	8	32	32	32
	Horse	-	-	4	16	16	16
	Monkey	-	-	8	16	16	32
JL-5	Turkey	8	-	-	-	8	32
	Sheep	16	-	-	2	16	32
	Horse	4	-	-	-	-	8
	Monkey	8	-	1[4]	2	8	32
Ur PT1	Turkey	4	2	8	4	32	16
	Sheep	16	1	8	16	64	32
	Horse	2	-	-	-	-	-
	Monkey	8	4	8	16	32	16
Ur PT3	Turkey	64	4	16	64	64	64
	Sheep	64	2	16	64	64	64
	Horse	64	-	-	16	32	32
	Monkey	64	16	16	64	128	64
Enders	Turkey	16	-	-	32	32	128
	Sheep	32	-	-	16	32	128
	Horse	16	-	-	8	16	64
	Monkey	16	4	1	16	16	64
Rubini	Turkey	64	16	-	4	32	32
	Sheep	64	16	-	4	32	64
	Horse	64	8	-	-	32	32
	Monkey	64	16	4	16	32	32
Po15/t	Turkey	4	-	4	16	-	-
	Sheep	16	32	16	16	64	8
	Horse	-	-	-	-	-	-
	Monkey	16	8	8	16	64	16

[1] RBC = Red blood cell

[2] P0 = Vero-grown parental virus

[3] - = Negative

[4] 1 = Neat only was positive for haemagglutination

B95a-derived and HeLa derived MuVs

In addition to increased infectivity titre, as measured by the $TCID_{50}$ assay, growth of the 7 MuVs in B95a or HeLa cells appeared to promote the production of progeny virus with identical or increased ability to cause haemagglutination in comparison to their parent viruses. The main example being JL-2, which was negative for haemagglutination prior to passage in B95a or HeLa cells (Tables 3.4A and B), but after passage caused haemagglutination up to 64 HAU.

No significant difference in HA activity was detected for MuVs, Ur PT3, Enders and Rubini after passage in B95a cells (Table 3.4A). Significant changes were detected for the other MuVs during passage in B95a cells (Table 3.4A), however, after the fifth passage, only the HA titre of JL-2 was significantly different to that of the Vero-grown parent with all four RBC types.

Significant decreases in HA activity were observed for all the MuVs after the initial passages in HeLa cells (Table 3.4B), corresponding to initial decrease in $TCID_{50}$/ml titre (Tables 3.2 and 3.3). The HA activity of all viruses then increased to a level not significantly different to the level of the respective parent virus, with the exception of JL-2. The JL-2 progeny had significantly higher HA activity than its parent virus.

After passage in B95a or HeLa cells, no haemagglutination was observed for Ur PT1 and Po15/t with horse RBCs.

BCL, CEF and MRC-5 derived MuVs

No haemagglutination activity was detected for any virus derived from passage in BCL, CEF or MRC-5 cells, with any of the erythrocyte species.

3.3.4 The RELATIONSHIP BETWEEN $TCID_{50}$ and HA

A $TCID_{50}$/HA ratio was calculated to determine if, over the five passages, there was any significant difference in the amount of $TCID_{50}$ units per HA unit. The relationship between $TCID_{50}$ and HA was determined after each passage of viruses in B95a and HeLa cells. The ratio was calculated by dividing the value for $TCID_{50}$ titre (Table 3.2) by an average value of HA titre (Table 3.4), excluding the values for horse RBCs because considerably lower results were observed with these erythrocytes with some viruses.

The value for the ratio of the parent viruses (Tables 3.5A and B) varied by > 3 log_{10} and was incalculable for JL-2, due to the absence of HA activity. However, after the fifth passage in B95a cells (Table 3.5A) the ratios were similar for all the viruses, ranging from 5.15 log_{10} to 5.91 log_{10}. After five passages in HeLa cells (Table 3.4B) the $TCID_{50}$/HA values were also higher than the original Vero-grown parental values, ranging from 4.08 log_{10} to 5.91 log_{10}.

Table 3.5A: Calculated ratio of $TCID_{50}$/HA for each virus passaged in B95a cells. Values are express in Log_{10}.

VIRUS	PASSAGE LEVEL					
	P0[1]	1	2	3	4	5
JL-2	-[2]	2.61	4.96	5.98	5.52	5.15
JL-5	3.69	-	-	4.23	4.59	5.30
Ur PT1	5.18	5.80	5.23	4.97	5.70	5.91
Ur PT3	5.20	4.83	4.32	3.26	3.56	5.26
Enders	3.78	4.54	3.58	4.34	5.08	5.61
Rubini	2.52	3.69	3.40	4.34	5.38	5.15
Po15/t	3.28	3.77	3.83	5.88	5.45	5.61

[1]P0, parental virus, prior to passage
[2]-, incalculable, due to the absence of HA activity

Table 3.5B: Calculated ratio of $TCID_{50}$/HA for each virus passaged in HeLa cells. Values are expressed in Log_{10}.

VIRUS	PASSAGE LEVEL					
	P0[1]	1	2	3	4	5
JL-2	-[2]	-	4.41	3.63	4.62	4.36
JL-5	3.69	-	3.28	4.78	5.26	5.43
Ur PT1	5.18	4.78	5.56	4.41	4.65	5.91
Ur PT3	5.20	4.04	4.53	4.89	5.43	4.08
Enders	3.78	2.26	3.28	3.57	5.11	4.85
Rubini	2.52	0.91	3.15	2.92	4.72	5.00
Po15/t	3.28	1.78	2.26	5.67	4.95	4.30

[1] P0, Parental virus, prior to passage
[2] -, incalculable, due to absence of HA activity

3.3.5 EFFECT of CELL PASSAGE on MuV NA ACTIVITY

Neuraminidase assays were performed as described in Chapter II. Neuraminidase activity was detected only for Vero-grown parental viruses Enders and Rubini; both had an NA titre of 8 (Table 3.6). Significant changes in NA activity between viruses were taken to be where a > 4-fold change was observed.

B95a-derived MuVs

In addition to increasing $TCID_{50}$ titres and HA titres, growth of the 7 MuVs in B95a cells appears to produce progeny viruses with an increased neuraminidase activity (Table 3.6), than their respective parent virus. After five passages in B95a cells, all the MuVs, except for Rubini, had increased NA in comparison to their Vero-grown parental viruses. The NA activity of Rubini was similar to its parental virus.

The NA activity of JL-2, JL-5 and Po15/t became detectable at the third passage (Table 3.6), the NA activity of Ur PT1 and Ur PT3 became detectable after the first and second passages respectively (Table 3.6) and remained positive thereafter. NA activity was detected in Enders and Rubini at each passage level. The NA titre of Enders virus, derived after three and five passages, had a NA titre of 128 NAU and 64 NAU respectively, the highest value of all viruses studied (Table 3.6).

HeLa-derived MuVs

Corresponding to the apparent adaptation period (as determined by the $TCID_{50}$ assay) for MuV growth in HeLa cells, NA activity was only observed after the third or fourth passage for all MuVs (Table 3.6). Thereafter, for most of the viruses, NA activity was retained at a similar level, with the exception of Po15/t, where NA activity was detected at its third passage only and not thereafter (Table 3.6). For all the MuVs, bar Rubini and Po15/t, the NA activity after the fifth passage was significantly higher than that of their parent virus.

Table 3.6: Neuraminidase assay results

CELL LINE	VIRUS	PASSAGE LEVEL					
		P0[1]	P1	P2	P3	P4	P5
B95a	JL-2	-[2]	ND[3]	-	4	4	1
	JL-5	-	ND	-	1	8	8
	Ur PT1	-	-	1	4	2	8
	Ur PT3	-	16	8	16	16	8
	Enders	8	16	32	128	16	64
	Rubini	8	32	16	16	8	16
	Po15/t	-	ND	-	2	8	8
BCL	JL-2	-	ND	-	-	-	-
	JL-5	-	ND	-	-	-	-
	Ur PT1	-	ND	-	-	-	-
	Ur PT3	-	ND	-	-	-	-
	Enders	8	ND	-	-	-	-
	Rubini	8	ND	-	-	-	-
	Po15/t	-	ND	-	-	-	-
CEF	JL-2	-	ND	-	-	-	-
	JL-5	-	ND	-	-	-	-
	Ur PT1	-	ND	-	-	-	-
	Ur PT3	-	ND	-	-	-	-
	Enders	8	ND	-	-	-	-
	Rubini	8	ND	4	-	-	-
	Po15/t	-	ND	-	-	-	-
HeLa	JL-2	-	ND	-	16	16	8
	JL-5	-	ND	-	-	16	8
	Ur PT1	-	ND	-	-	4	4
	Ur PT3	-	ND	-	8	8	8
	Enders	8	ND	-	8	16	64
	Rubini	8	ND	-	2	32	32
	Po15/t	-	ND	-	8	-	-
MRC-5	JL-2	-	ND	-	-	-	-
	JL-5	-	ND	-	-	-	-
	Ur PT1	-	ND	-	-	-	-
	Ur PT3	-	ND	-	-	-	-
	Enders	8	ND	-	-	-	-
	Rubini	8	ND	4	1	-	-
	Po15/t	-	ND	-	-	-	-

[1] P0, Vero-grown parental virus
[2] -, Negative
[3] ND, Not done

BCL, CEF and MRC-5 -derived MuVs

No NA activity was detected for JL-2, JL-5, Ur PT1, Ur PT3, Enders and Po15/t derived from passage in BCL, CEF or MRC-5 cells (Table 3.6).

No NA activity was detected either for Rubini derived from passage in BCL cells. However, NA activity was detected for Rubini, after the second passage in CEF cells only and after the second and third passage in MRC-5 cells only but thereafter was not detected (Table 3.6). The detected activity was likely remaining from input virus.

3.3.6 The RELATIONSHIP BETWEEN $TCID_{50}$ and NA ACTIVITY

A $TCID_{50}$/NA ratio was calculated to determine if, over the five passages, there was any significant difference in the amount of $TCID_{50}$ units per NA unit. The ratio of $TCID_{50}$/NA was calculated for each virus grown in B95a and HeLa cells by dividing the $TCID_{50}$/ml (Table 3.2) by the NA titre (Table 3.6).

The value of the ratio for the parental viruses was incalculable due to the absence of NA activity, with the exception of Enders and Rubini (Table 3.7A and B). After five passages in B95a and HeLa cells, the amount of $TCID_{50}$ units per NA unit for each of the MuVs ranged from 5.15 $\log_{10}$ to 6.51 $\log_{10}$ in B95a cells and from 4.53 $\log_{10}$ to 6.63 $\log_{10}$ in HeLa cells (Table 3.7A and B), with the exception of Po15/t grown in HeLa cells, where the ratio was incalculable due to an absence of NA activity.

Table 3.7A: Ratio of $TCID_{50}$/NA for each virus passaged in B95a cells. Values are expressed in Log_{10}.

VIRUS	PASSAGE LEVEL					
	P0[1]	P1	P2	P3	P4	P5
JL-2	-[2]	-	-	6.46	6.34	5.86
JL-5	-	-	-	5.26	5.20	6.04
Ur PT1	-	-	6.28	5.49	6.52	6.52
Ur PT3	-	5.43	5.43	4.23	4.38	6.15
Enders	4.20	4.78	3.56	4.18	5.08	5.62
Rubini	3.41	3.81	4.00	3.57	5.68	5.15
Po15/t	-	-	-	6.70	6.28	5.68

[1] P0, Vero-grown parental virus
[2] -, Incalculable due to absence of neuraminidase activity

Table 3.7B: Ratio of $TCID_{50}$/NA for each virus passaged in HeLa cells. Values are expressed in Log_{10}.

VIRUS	PASSAGE LEVEL					
	P0[1]	P1	P2	P3	P4	P5
JL-2	-[2]	-	-	3.93	4.65	4.53
JL-5	-	-	-	-	5.08	5.32
Ur PT1	-	-	-	-	5.68	6.64
Ur PT3	-	-	-	5.80	6.46	5.98
Enders	4.20	-	-	4.00	5.34	5.08
Rubini	3.41	-	-	3.52	4.72	5.15
Po15/t	-	-	-	5.98	-	-

[1] P0, Vero-grown parental virus
[2] -, Incalculable due to absence of neuraminidase activity

3.4 DISCUSSION

In this study, seven MuV strains have been passaged five times in five different cell lines to increase our understanding of the effect of host cell passage on MuVs. The infectivity of the progeny from these passages was determined using the $TCID_{50}$ assay. The HA and the NA assays were used to determine whether non-infectious virus particles were present, which were not detected by the $TCID_{50}$ assay.

3.4.1 CHOICE OF CELL LINE

To increase the understanding of host cell passage, MuVs were grown in a variety of different cell substrates. The cell lines chosen for use in this study were B95a, BCL, CEF, HeLa and MRC-5 cells.

B95a cells are a marmoset lymphoid cell line transformed with EBV and have previously been shown to support the growth of other paramyxoviruses, such as measles virus (MV) (Kobune *et al.*, 1990). Indeed, MV grown in B95a cells was more representative of MV circulating among humans than MV grown in Vero cells (Kobune *et al.*, 1990). Rinderpest virus (RPV) and canine distemper virus (CDV), other paramyxoviruses of the *morbillivirus* genus, have also been grown in B95a cells (Kobune *et al.*, 1991; Kai *et al.*, 1993); again, the B95a-derived virus was more representative of the parental population than Vero-derived virus. Measles virus, RPV and CDV grown in B95a cells retained virulence for monkeys, rabbits and dogs respectively, whereas Vero grown virus became less virulent. Hence, growth of MuV in these cells was investigated to determine if B95a-derived virus differed from Vero-derived virus.

BCL cells are a murine lymphocytic cell line. Studies of the replication of MuV in murine cells (Tsurudome *et al.*, 1984) showed that full replication of MuV was possible despite other reports that propagation of MuV in murine cells led to abortive infections (Cantell *et al.*, 1961). The study by Tsurudome *et al* (1984) also suggested that murine cells of

lymphoid origin supported growth better than other murine derived cells. The BCL cells used in this project were interferon (IFN) negative (A. Meager, personal communication); this property possibly increased the potential for productive replication of MuVs in these cells because IFN, produced by cells in response to a virus infection, acts to eliminate virus.

Chicken embryo fibroblast (CEF) cells were used in this project because JL-2, JL-5, Ur PT1 and Ur PT3 had previously been exposed to these cell lines in the original production of the JL and Urabe live attenuated vaccine strains. Hence, it was likely that these viruses and the other MuVs would propagate on these cells. Also, growth of Urabe in these cells affected its phenotypic and genotypic characteristics (Afzal *et al.*, 1998); therefore, it was of interest to determine if growth in CEF cells affected the various other MuV strains.

HeLa cells are of human tumour origin and were originally epithelial cells. Epithelial cells are the sites of MuV infection of humans (Feldman *et al.*, 1989) and potentially the viruses could propagate efficiently in this cell line.

MRC-5 cells are diploid cells of foetal human lung origin and were used in this project because the Rubini strain had been propagated on these cells to produce the vaccine, and it was hoped that other MuVs might also grow in these cells.

3.4.2 CYTOPATHIC EFFECT

Cytopathic effect is defined as the histological appearance of damage produced in cultured cells by an infecting virus and is an indication of virus infectivity for the cell line. CPE manifested itself as cell death and cell fusion; enlarged cell formations with many nuclei, known as syncytia after passage in B95a and HeLa cells. CPE was not observed in BCL, CEF or MRC-5 cells infected with MuV, suggesting that no active replication was occurring. CPE could have developed in these cell lines after further passage and adaptation of the viruses to the cells. The extent of CPE differed with less cell fusion and

no production of spindle cells occurring in B95a or HeLa cells infected with Ur PT1 or Ur PT3, unlike infection with the other viruses. The protein most likely to manifest these observations is the F protein, which is responsible for virus-to-cell fusion (fusion from without) and cell-to-cell fusion (fusion from within). It is also likely that the HN protein plays an equal role in cell fusion.

3.4.3 PROPAGATION of MuV in ALTERNATE CELL LINES

One measure of virus infectivity is the $TCID_{50}$ assay. $TCID_{50}$ assays were performed using Vero cells; hence, if viruses were produced after passage, which were not infectious for Vero cells, they would not be detected in the assay. Therefore, HA and NA assays were used in addition to determine if such virus was present. The results of this chapter are summarised in Table 3.8.

The $TCID_{50}$ results indicate three outcomes from propagating MuV in alternate cell lines:

- A decrease in infectivity, followed by an increase, suggesting an adaptation event (JL-5 in B95a cells, all MuVs in HeLa cells and Ur PT3 in MRC5 cells).
- An increase in virus infectivity with no prior decrease (all viruses in B95a cells, except JL-5), suggesting an increasing fitness for the cell line.
- A decrease in virus infectivity (MuVs in BCL, CEF and MRC-5 cells (except Ur PT3)).

For growth of JL-5 in B95a cells (Figure 3.4A), all the MuVs in HeLa cells (Figure 3.4D) and Ur PT3 in MRC-5 cells (Figure 3.4E), an apparent period of adaptation was required. Adapted viruses possibly contain a change in a viral protein giving them an advantage to replicate better than the parent in the selecting cell substrate. This change could be located within either of the two envelope glycoproteins because these proteins play a crucial role in virus attachment and entry to the cell. It is also possible that a change could be located within any of the other proteins.

Table 3.8: Summary of the results of Chapter III. Presented are the significant differences observed between Vero-grown parental viruses and their progeny after five passages in alternate cell lines.

CELL	Virus	Log_{10} $TCID_{50}$	HA	NA	TCID/HA	TCID/NA
B95a	JL-2	+[1]	+	+	+	+
	JL-5	+	+	+	+	+
	Ur PT1	+	+	+	=	+
	Ur PT3	+	=[3]	+	+	+
	Enders	+	=	+	+	+
	Rubini	+	=	=	+	+
	Po15/t	+	=	+	+	+
BCL	JL-2	-[2]	=	=	NA[4]	
	JL-5	-	-	=		
	Ur PT1	-	-	=		
	Ur PT3	-	-	=		
	Enders	-	-	-		
	Rubini	-	-	-		
	Po15/t	-	-	=		
CEF	JL-2	-	=	=	NA	
	JL-5	-	-	=		
	Ur PT1	-	-	=		
	Ur PT3	-	-	=		
	Enders	-	-	-		
	Rubini	-	-	-		
	Po15/t	-	-	=		
HeLa	JL-2	+	+	+	+	+
	JL-5	+	=	+	+	+
	Ur PT1	+	=	+	=	+
	Ur PT3	=	=	+	=	+
	Enders	+	+	+	+	=
	Rubini	+	=	=	+	+
	Po15/t	=	=	+	+	+
MRC-5	JL-2	-	=	=	NA	
	JL-5	-	-	=		
	Ur PT1	-	-	=		
	Ur PT3	=	-	=		
	Enders	-	-	-		
	Rubini	-	-	-		
	Po15/t	-	-	=		

[1] +, Significant increase in comparison to Vero-grown parental virus
[2] -, Significant decrease in comparison to Vero-grown parental virus
[3] =, No significant change in comparison to the Vero-grown parental virus
[4] NA, Not applicable

Growth of all MuVs, except for JL-5, in B95a cells did not require an adaptation period and progeny virus increased in infectivity after passage in B95a cells in comparison to the Vero-grown parent, implying that growth of MuVs in B95a cells enhances virus infectivity, likely due to host cell factors. The ability of a virus to replicate in a host cell is known as viral fitness and results from this Chapter suggest an increase in viral fitness after passage in B95a and HeLa cells.

Although different inoculum volumes and adsorption periods were used to try to improve virus growth in BCL, CEF or MRC-5 cells, in most cases little infectious virus was produced. It is likely that the minimal virus activity detected was remaining inoculum, due to the method used to harvest the virus, which was freeze-thawing as opposed to collecting the supernatant. Whilst growing MuVs in these cell lines it may have been beneficial, not only to increase the MOI further, but to treat the cells prior to inoculation with substances such as polybrene, which disturb the charge on the cell membrane, disrupting the permeability of the membrane and allowing greater opportunity for virus entry to cell. For active replication of MuV to occur the F protein has to be cleaved by a protease into its fusogenic form. Therefore, it is possible that these cells did not contain the protease enzyme necessary for cleavage of the F protein into its active form; thus, virus progeny would only be capable of one round of infection. The addition of a protease in the inoculum could ensure cleavage of the F protein into its fusogenic form, possibly enabling further rounds of replication. Influenza virus is another example of a virus, which also requires a protease to enable productive replication *in vivo* or *in vitro*. Reports, regarding the growth of influenza virus in MRC-5 cells, have indicated that optimal growth conditions include the addition of trypsin into the medium, during the inoculation period (Reina *et al.*, 1997 & Herrero-Uribe *et al.*, 1983), hence, the addition of trypsin, whilst trying to grow MuV in the different host cells may have been valuable.

For productive replication to occur, influenza virus requires proteolytic cleavage of its HA by a cellular protease, of which furin is one example (Rott *et al.*, 1995). Furin, a member of the subtilisin family, recognises a multibasic cleavage signal on the HA of influenza viruses, where it cleaves the HA into its fusogenic form, thus enabling active replication to occur. Serine proteases, which are only secreted from a restricted number of cell types, recognise a monobasic cleavage signal (Rott *et al.*, 1995) and lead to only a localised infection. However, serine proteases have been observed to have multiple activities in HIV-1 infection of macrophages and increase macrophage susceptibility to acute infection by HIV-1 (Moriuchi *et al.*, 2000).

In general, reactivity of the viruses with equine RBCs was lower than with the other RBCs, to the extent that neither Ur PT1, Po15/t nor their cell-derived progeny were able to agglutinate horse erythrocytes, except for Vero-derived parental Ur PT1, which only had a titre of 2. This suggested some common feature between the HN proteins of these two strains which differed from the others. Erythrocytes from chickens, humans and sheep contain both SAα2,3Gal- and SAα2,6Gal-specific lectin-reactive oligosaccharides. In contrast, horse erythrocytes contain virtually 100% SAα2,3Gal linkages (Ito *et al.*, 1997) and lack of haemagglutination of horse RBCs by Ur PT1 and Po15/t could be caused by their inability to recognise the SAα2,3Gal linkage. While JL-2, JL-5, Ur PT3, Enders and Rubini mainly recognise erythrocytes containing the SAα2,6Gal linkage, they can also recognise the SAα2,3Gal linkage. This difference in recognition of erythrocytes is likely to be due to differences in the receptor-binding site within the HN protein in the different viruses.

Increases in HA titre were observed for MuVs grown in B95a and HeLa cells and correspond to an increase in $TCID_{50}$. In contrast, virus derived from passage in BCL, CEF or MRC-5 cells could not haemagglutinate. By comparing $TCID_{50}$/HA ratios, significant differences indicate changes in the ability of a virus to grow in B95a or HeLa cells could

be detected. A low ratio could indicate the presence of non-infectious particles ($TCID_{50}$ value low but HA detectable), whereas high ratios imply a relative lack of non-infectious particles in the virus strains. The $TCID_{50}$/HA ratios suggest B95a and HeLa cells are suitable cell lines for growing MuVs since after five passages, high $TCID_{50}$ titres and $TCID_{50}$/HA ratios were obtained (Tables 3.2 and 3.4). The process by which passage of MuV in B95a or HeLa cells selected for a fitter virus is unknown. However, these higher ratios obtained after five passages of MuV in B95a or HeLa cells indicate that more efficient replication is occurring in later passages.

Results obtained in this study regarding the $TCID_{50}$/HA ratio are in agreement with a previous study by Isaacs & Donald (1955). Isaacs and Donald showed the ratio of egg infective dose 50 (ID 50) to agglutinating dose for MuV ranged from 4.5 $\log_{10}$ to 6.3 $\log_{10}$. Results from this study show nearly identical results where the $TCID_{50}$/HA ratios for MuV growth in B95a and HeLa cells ranged from 4.08 $\log_{10}$ to 5.91 $\log_{10}$. Isaacs and Donald also presented data indicating that MuV has a low efficiency of initiating infection, needing some hundreds of particles/ID 50, in comparison to other haemagglutinating animal viruses, which require approximately 10 particles/ID_{50}. The authors suggested that the low efficiency of replication observed by MuVs was because this virus was not readily absorbed by the RBCs and therefore at the agglutination end point only a small fraction of MuV was absorbed to the RBCs. A study by Desselberger (1975), produced similar results to the study by Isaacs and Donald, where approximately 20 influenza A virus particles were observed to correspond to one egg ID 50.

The HN glycoprotein of MuV not only allows the virus to bind to and agglutinate erythrocytes from certain species but also possesses neuraminidase activity, which removes the terminal sialic acid from glycoproteins and glycolipids present on the surface of cells, thus allowing efficient budding of the virus from the cell and preventing self-aggregation of the budded virus particles. Neuraminidase activity was detected only for parental

MuVs, Enders and Rubini. It is likely that Enders and Rubini have a genetic difference in the neuraminidase active site within the HN gene, which accounts for their NA activity in comparison to the other viruses. The level of neuraminidase activity of each parental virus and its cell-derived progeny were investigated and differences were observed. After passage in B95a and HeLa cells, the NA activity became detectable for all viruses, increasing as infectivity titres increased.

It has been previously proposed that the level of NA activity affected MuV cell fusion (Merz and Wolinsky, 1981) and this could affect plaque morphology. Since neuraminidase activity is necessary to release virus effectively from the infected cell, a higher level of NA could lead to a greater release rate, leading to a larger plaque morphology. It is also possible that the NA activities are not at a high enough level to affect plaque morphology alone and that other factors are involved such as the F protein, which affects cell fusion or HA activity which could also affect plaque morphology.

3.4.4 CONCLUSION

The highest infectivity, HA and NA titres were observed for B95a or HeLa-derived MuVs. MuV growth in B95a cells resulted in the production of higher quality virus without any apparent need for adaptation, except for JL-5. In contrast, virus growth in HeLa cells did not produce virus of such high quality and there was a more obvious period of adaptation. It is likely that viruses, which showed an adaptation period, will contain a difference in genotype and/or phenotype. Virus, which grew without a period of adaptation, is less likely not to contain a difference in genotype or phenotype.

Since the data suggests that variants of the original MuV population were possibly propagated after the passage of MuV in HeLa cells, and that B95a-derived virus may be identical to the parent virus, changes in other phenotypic properties of the viruses may also

be apparent. Therefore, the progeny viruses were analysed further by investigating plaque morphology and antigenic profiles using a panel of MAbs.

CHAPTER IV

PHENOTYPIC CHARACTERISATION OF MuV

4.1 INTRODUCTION

Results from the previous chapter indicate that B95a- or HeLa-derived MuV is of higher fitness, identified by an increase in infectivity titre, compared to the Vero-grown parental virus. Therefore, B95a and HeLa cell lines appear to be suitable alternatives to Vero cells as a source of infectious MuV. To characterise further the viruses derived from the alternate cell lines, the phenotypic properties of plaque morphology and antigenic profile were compared to those of the Vero-grown parental virus.

The plaque morphology of a virus is directly related to its replicative ability. The results from Chapter III indicate an increasing ability to produce infectious progeny after passage in B95a or HeLa cells. Therefore, plaque morphology was assessed to investigate if changes occurred due to passage in alternate cell lines. Plaque assays for MuV have previously been and are routinely performed in laboratories at NIBSC using Vero cells and a further aim was to investigate alternative cell lines for use in these assays.

In this instance, virus antigenic profiles are related to the properties of the HN protein because the monoclonal antibodies used were raised against this protein. Results from Chapter III indicate increases in the ability of the viruses to cause haemagglutination and increases in neuraminidase activity. These increases in activity could be caused by changes in the HN protein, which could, therefore, affect the antigenic properties of the HN protein.

4.2 EXPERIMENTAL PROCEDURES

Initially, plaque assays were performed on the parental Vero-adapted MuVs using either B95a, CEF, HeLa, MRC-5 or Vero cells (as described in Chapter II) to determine the cell line of choice for use in further plaque assays. BCL cells grow in suspension and do not form monolayers, hence, by definition they were excluded from this particular study. Once the cell substrate for use in plaque assays had been determined, the plaque morphology of

parental viruses was examined and used as a basis for the comparison of those formed by virus derived from passage in the alternate cell lines.

To determine virus antigenic profiles, neutralisation assays (as described in Chapter II) were performed with a panel of anti-HN monoclonal antibodies (MAbs). The MAbs used had previously been characterised for neutralisation activity, neuraminidase inhibitory and haemagglutination inhibitory activities at NIBSC (Yates *et al.*, 1996) and were known as MAbs 1637, 1641, 1648, 1689, 1721, 1726 and 1970 (Table 4.1). Neutralisation assays were performed by incubating each MAb (at a dilution of 1 in 100 from the stock) with 500 $TCID_{50}$/ml of virus (or virus was used neat if its titre was lower than 500 $TCID_{50}$/ml) at 4°C for 90 minutes and then incubating 50µl of this virus/MAb mixture on Vero cells for 1-2 hours in a 24 well plate, when the medium was replaced with CMC containing MAb. The plates were then incubated at 35°C for 7 days (See Chapter II for further details).

Table 4.1: Characterisation of monoclonal antibodies: biological activity with the Urabe strain of mumps (Yates *et al.*, 1996).

MAB	NEUTRALISATION TITRE	HI TITRE	NI TITRE
1637	100 000	<20	500
1641	1 000	<20	500
1648	10 000	<20	1 000
1689	100 000	>2560	100
1721	100 000	5 000	>100
1726	10 000	1 280	>100
1970	100 000	>20 480	>100

Using these MAbs, the antigenic profiles of Vero-grown parental viruses and viruses derived from passage in the alternate cells lines were studied and compared, as described in Chapter II.

4.3 RESULTS

4.3.1 PLAQUE ASSAYS

Cell substrate for use in plaque assays

Initially, the plaque morphologies of the Vero-grown parental MuVs were investigated using Vero, B95a, CEF, HeLa and MRC-5 cells under a CMC overlay (as described in Chapter II) to determine the most suitable cell line for use in future assays. After a 7 day incubation period, no plaques were observed in the assays using CEF or MRC-5 cells. Although faint plaques were observed in assays using B95a and HeLa cells, the cells did not survive under CMC or agar overlay for the 7 days required for a MuV plaque assay. Plaques were observed with the use of Vero cells and the cell monolayer survived intact. It was concluded that Vero cells were the most appropriate cell substrate with which to perform subsequent plaque assays.

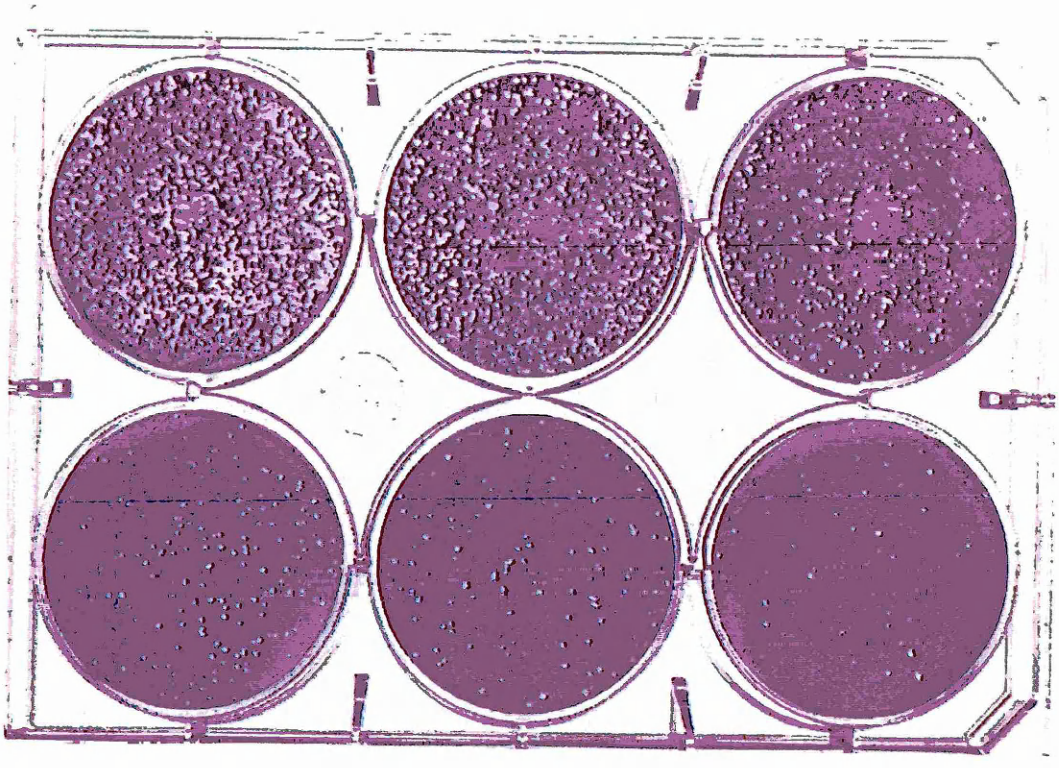
Plaque morphology of parental MuV in Vero cells.

The plaque morphology of each virus was assessed by measuring the diameter of the plaque.

It has previously been established that JL-2 and JL-5 strains are distinguishable from one another by their plaque morphology in Vero cells (Afzal *et al.*, 1997). In this study, JL-2 formed smaller plaques when compared to those of JL-5 (Figure 4.1), in agreement with this previous study. Ur PT1 and Ur PT3 are also distinguishable by their plaque morphology upon infection of Vero cells; Ur PT1 plaques are larger and clearer than those of Ur PT3 (Afzal *et al.*, 1998), and identical results were obtained in this study (Figure 4.2). Results from this study also indicated that Enders and Rubini had distinguishable plaque morphology; Enders formed larger and clearer plaques than those of Rubini (Figure 4.3). Plaques formed by Po15/t were intermittent in size between those of JL-5 and Ur PT3 (Figure 4.4).

Figure 4.1: The different plaque morphologies of a) JL-2 and b) JL-5. Plaques formed by JL-2 are smaller than those formed by JL-5

a)



b)

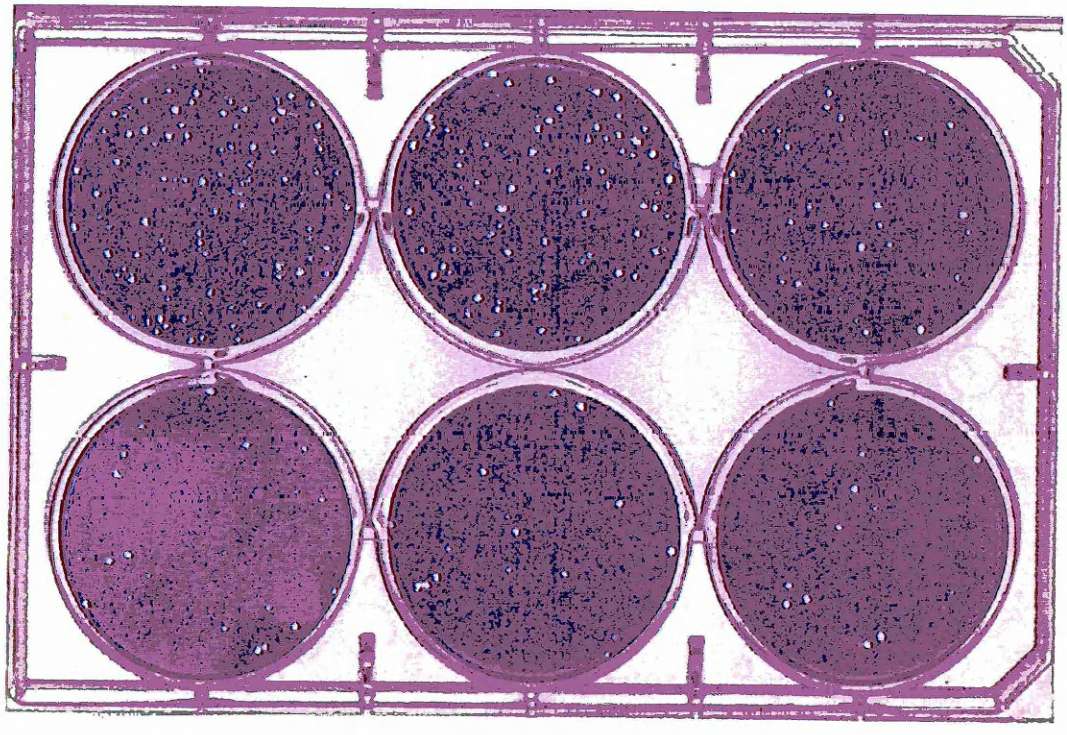
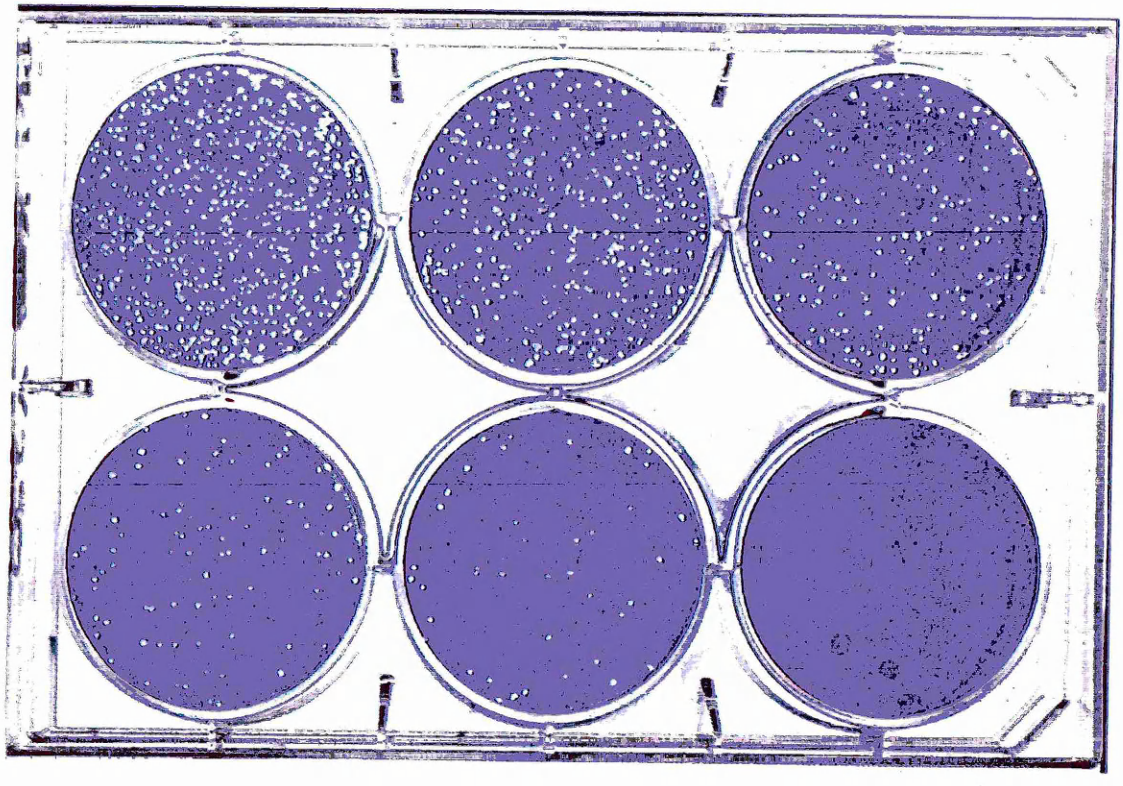


Figure 4.2: The different plaque morphologies of a) Ur PT1 and b) Ur PT3. Plaques formed by Ur PT1 are larger and clearer than those formed by Ur PT3.

a)



b)

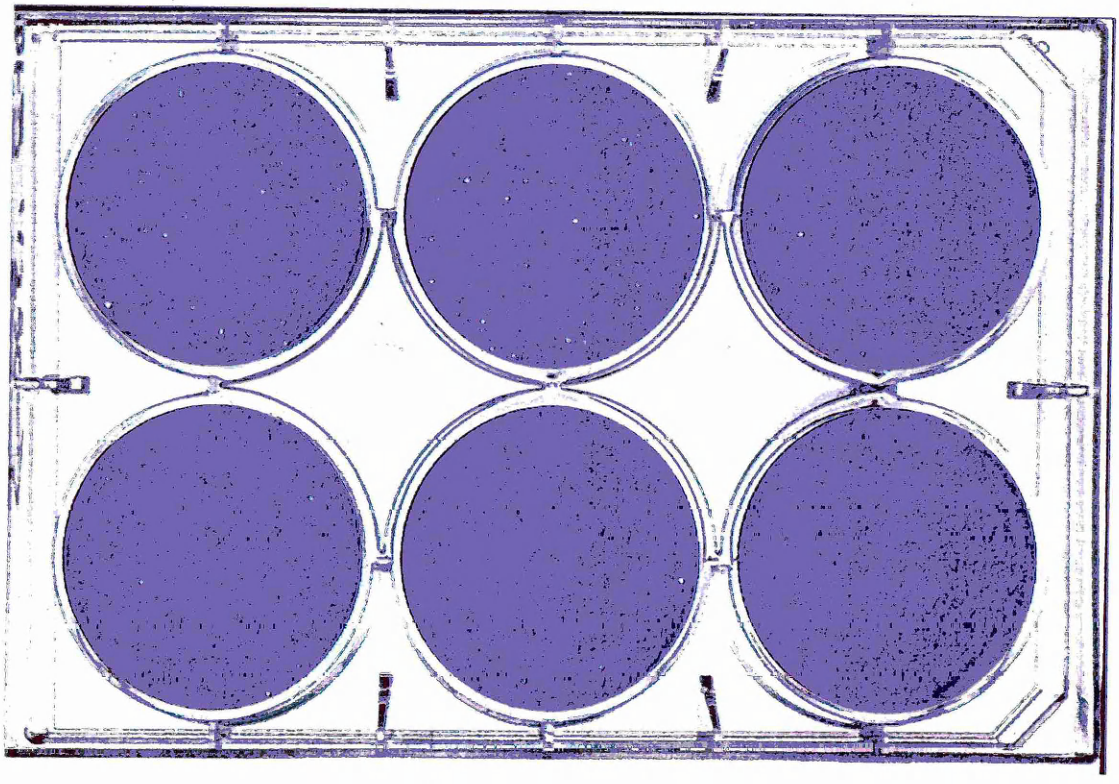
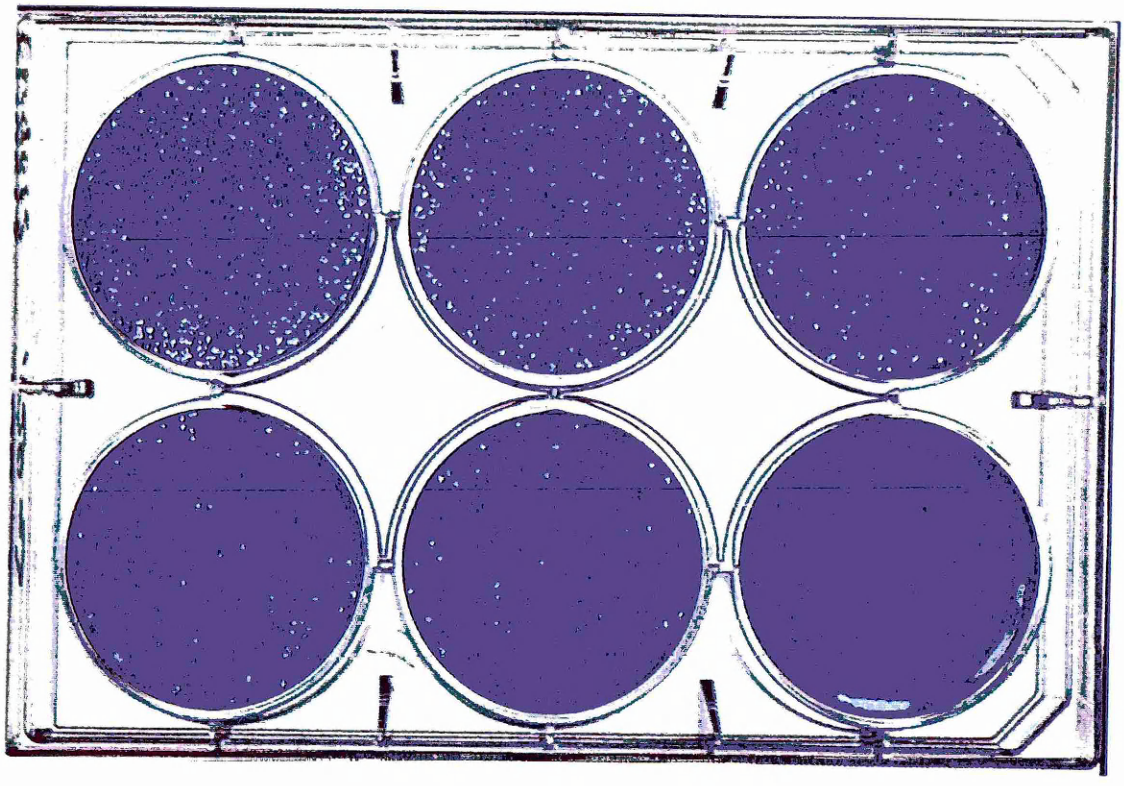


Figure 4.3: The different plaque morphologies of a) Enders and b) Rubini. Plaques formed by Enders were slightly larger and clearer than those formed by Rubini.

a)



b)

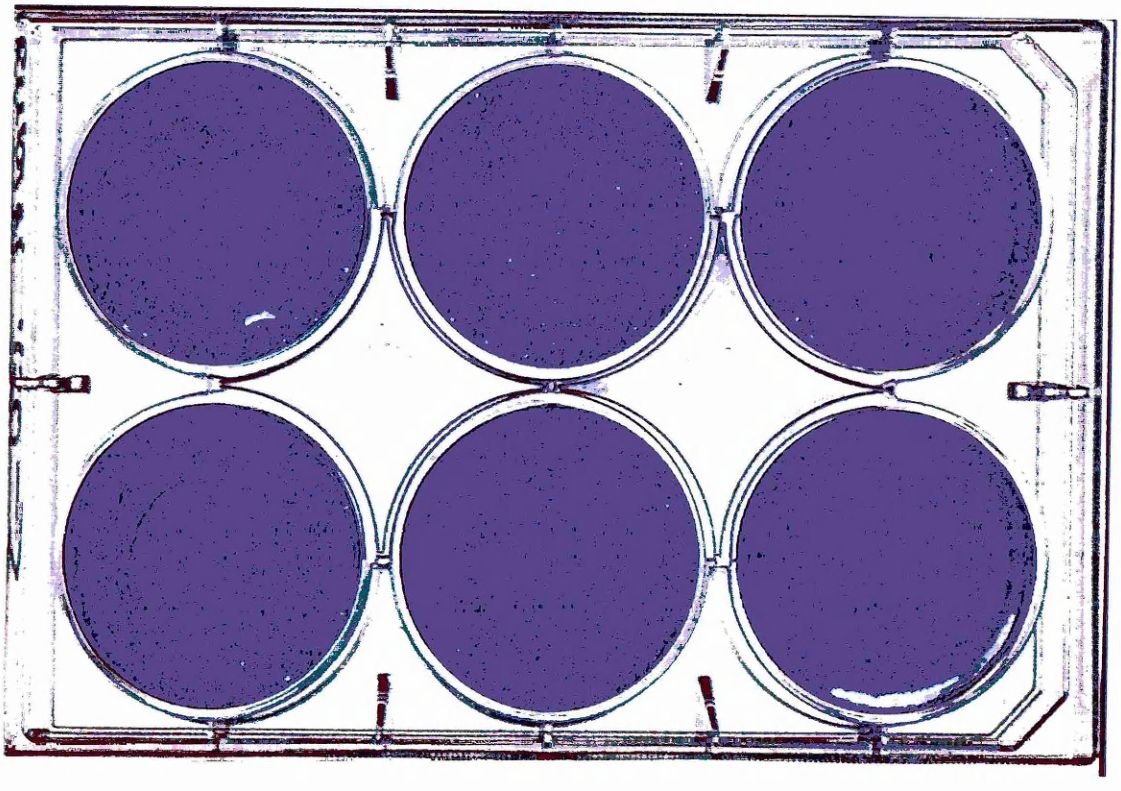
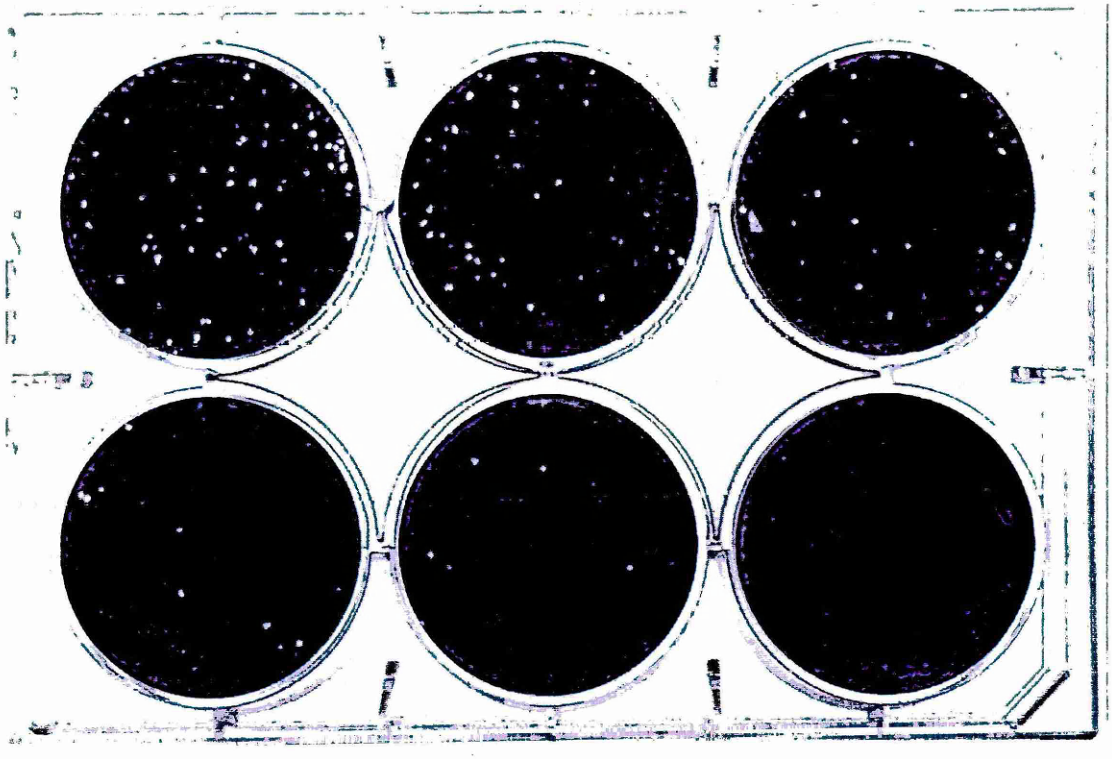


Figure 4.4: The plaque morphology of Po15/t.



Effect of host cell passage on plaque formation

Differences in plaque morphology were observed for JL-2, derived by one or more passages in B95a cells, where smaller plaques were formed in comparison to those formed by its Vero-grown parental virus (Figures 4.1A and 4.5). A difference in plaque morphology for Ur PT3 in HeLa cells was observed only after the fifth passage when larger and clearer plaques were formed (Figures 4.6 and 4.2B). A difference in plaque morphology for Enders in HeLa cells was only observed after the second passage when smaller plaques were formed when compared to those of the Vero-grown parental virus (Figures 4.7 and 4.3A). A change in plaque morphology was only observed for Rubini in B95a cells after the fifth passage when clearer plaques were formed in comparison to those of lesser-passaged virus (Figures 4.8 and 4.3B).

The plaques formed by MuVs after passage in all the other cell lines were identical in morphology to those of the Vero-grown parental virus.

Table 4.2 summarises the different plaque morphologies.

Table 4.2: Summary of the different plaque morphologies of MuV.

VIRUS	CELL LINE					
	Vero[1]	B95a	BCL	CEF	HeLa	MRC-5
JL-2	Clear, 1mm[2]	Clear, 0.5mm	-	-	-	-
JL-5	Clear, 1.5mm	-[3]	-	-	-	-
Ur PT1	Clear 1mm	-	-	-	-	-
Ur PT3	Cloudy, 0.5mm	-	-	-	Cloudy, 1mm	-
ENDERS	Clear, 0.5mm	-	-	-	Clear, <0.5mm	-
RUBINI	Cloudy, 0.5mm	Clear, 0.5mm	-	-	-	-
Po15/t	Clear, 1mm	-	-	-	-	-

[1] = Cell line in which the viruses were grown to produce the Vero-grown parental viruses
[2] = Plaque diameter
[3] = Plaque morphology is identical to that formed by the parental virus

Figure 4.5: The plaque morphology of JL-2 passaged in B95a cells. The plaques formed were smaller than the parental JL-2 plaques.

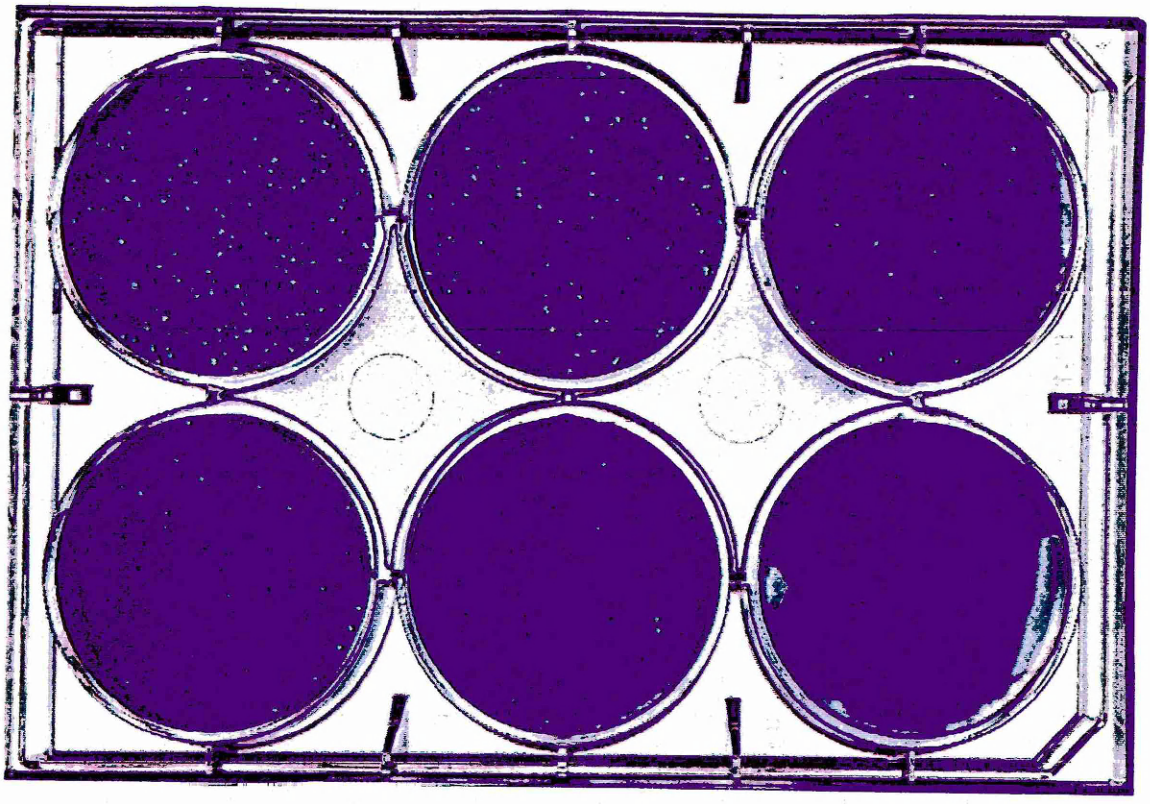


Figure 4.6: The plaque morphology of UrPT3 passaged in HeLa cells. The plaques formed were less cloudy than the parental Ur PT3 plaques.

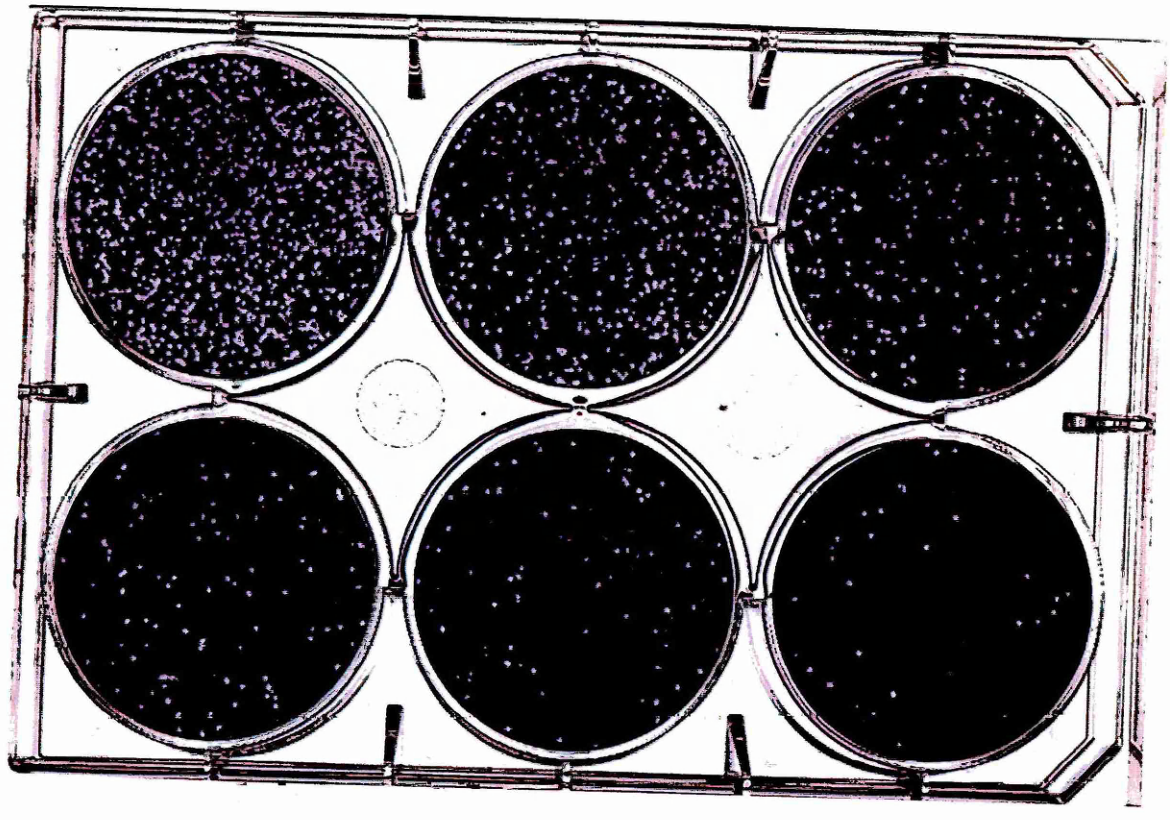


Figure 4.7: The plaque morphology of Enders passaged in HeLa cells. The plaques formed were smaller than the parental Enders plaques.

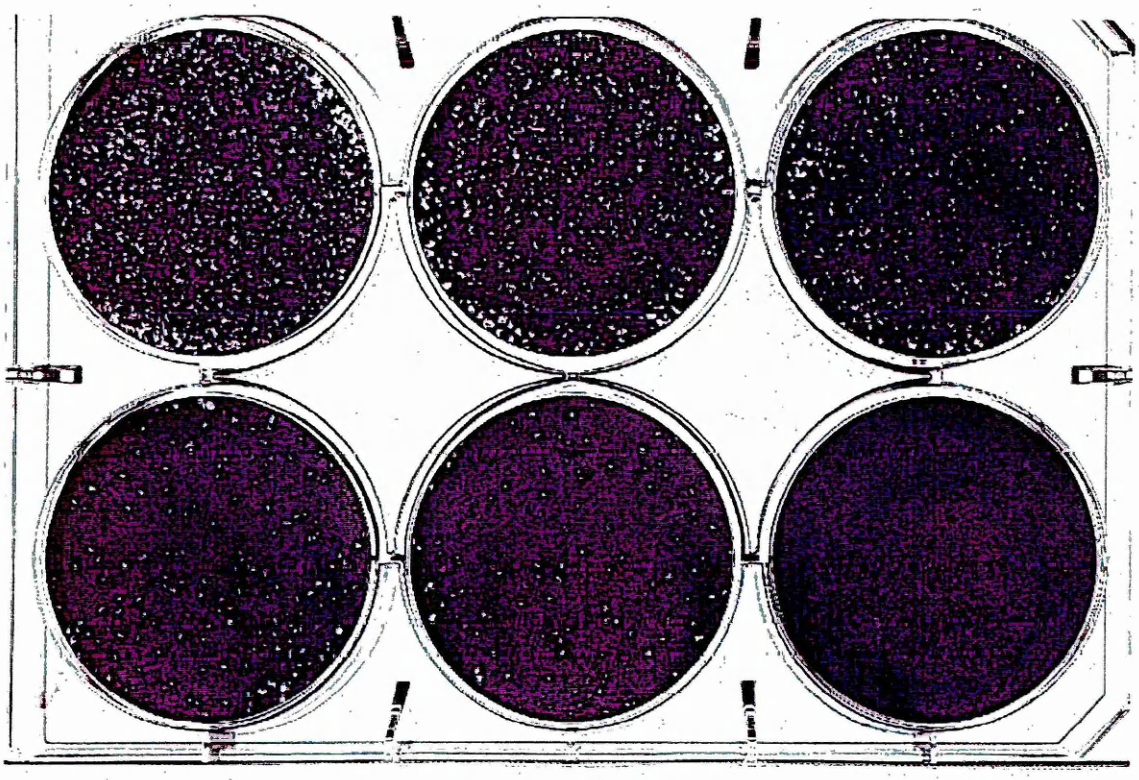
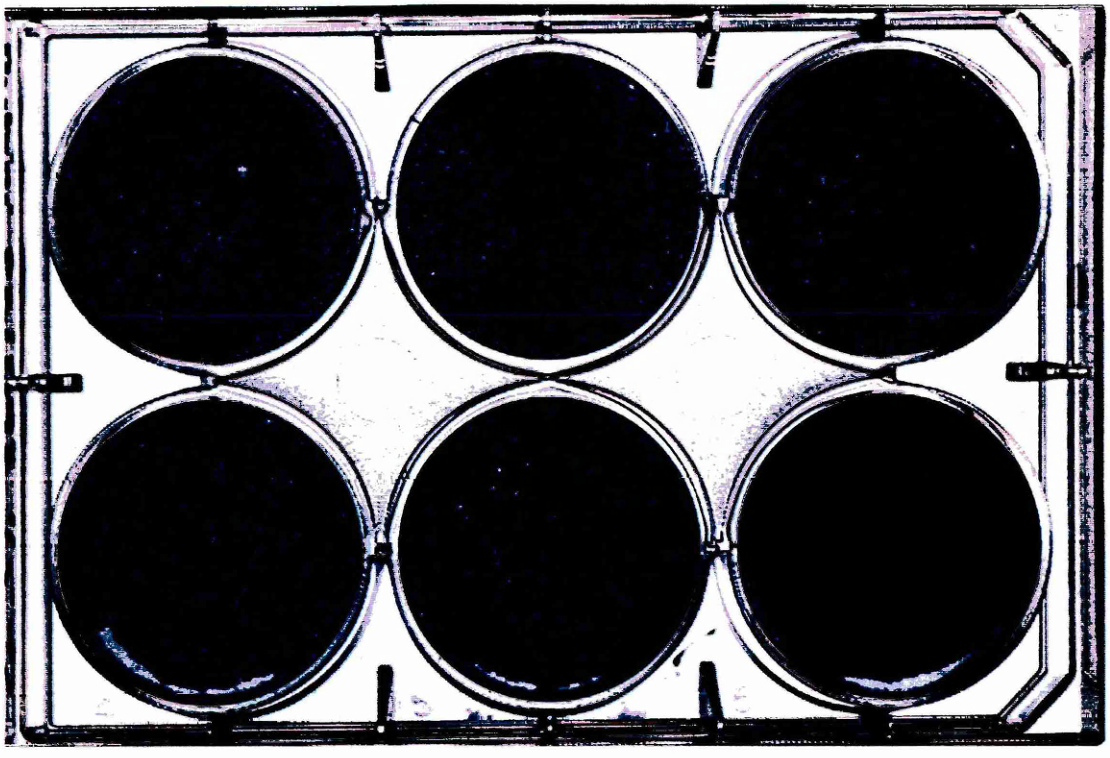


Figure 4.8: The plaque morphology of Rubini passaged in B95a cells. The plaques formed were slightly clearer than the parental Rubini plaques.



To investigate reversion of plaque morphology, JL-2, derived by two passages in B95a cells, was passaged four times in Vero cells. This virus also formed smaller plaques than the Vero-grown parental JL-2 virus (data not shown); therefore no reversion was detected.

4.3.2 ANALYSIS of the ANTIGENIC PROFILE of MuV

The antigenic profiles of MuVs were assessed by determining the ability of a panel of MAbs to neutralise virus infectivity (Table 4.1). Virus neutralisation (sensitivity) was observed by a complete absence of plaque formation, whereas resistance is indicated by the formation of plaques.

Results of the neutralisation assays using 7 MAbs (Table 4.3) indicated that two of the MAbs (1637 and 1648) were active against the Vero-grown parental JL-2 and three against parental JL-5 (1637, 1648 and 1970). Vero-grown parental Ur PT1 and Ur PT3 were neutralised by six of the MAbs but not by MAb 1637 (Table 4.3). Vero-grown parental Enders, Rubini and Po15/t were also neutralised by six MAbs but were resistant to MAb 1970 (Table 4.3).

To investigate differences in antigenic profile between Vero-grown parental virus and the alternate-cell-derived viruses, neutralisation assays were performed on viruses derived by five passages in each cell type and the results compared to the antigenic profile of the Vero-grown parental virus.

JL-2 (sensitive to MAb 1648) became resistant to MAb 1648 after five passages in B95a cells. The results of the neutralisation assay also revealed that B95a-derived JL-2, although retaining resistance to MAbs 1689, 1726 and 1970, formed smaller plaques in the presence of the MAbs compared to in their absence (Table 4.3). The formation of smaller plaques in the presence of a MAb was also observed with HeLa-derived JL-2 with MAb 1970 (Table 4.3). JL-5 (sensitive to 1648) became resistant to MAb 1648 after five

Table 4.3: Reactivity of a panel of MAbs with the parental viruses, JL-2, JL-5, Ur PT1, Ur PT3, Enders, Rubini and Po15/t and their progeny after five passages in the cell line indicated. The neutralisation titres of the MAbs are presented in Table 4.1.

VIRUS	CELL	MONOCLONAL ANTIBODY						
		1637	1641	1648	1689	1721	1726	1970
JL-2	Vero[1]	S[2]	R[3]	S	R	R	R	R
	B95a	-[4]	-	R	*[5]	-	*	*
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	-	-	-	-	*
	MRC-5	-	-	-	-	-	-	-
JL-5	Vero	S	R	S	R	R	R	S
	B95a	-	-	-	-	-	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	R	-	*	*	-
	MRC-5	-	-	-	-	-	-	-
Ur PT1	Vero	R	S	S	S	S	S	S
	B95a	-	-	-	-	-	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	*	R	-	R	R	-
	MRC-5	-	-	-	-	-	-	-
Ur PT3	Vero	R	S	S	S	S	S	S
	B95a	-	-	-	-	-	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	-	-	-	-	-
	MRC-5	-	-	-	-	-	-	-
Enders	Vero	S	S	S	S	S	S	R
	B95a	-	-	-	R	-	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	-	R	-	-	-
	MRC-5	-	-	-	-	-	-	-
Rubini	Vero	S	S	S	S	S	S	R
	B95a	-	R	-	-	R	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	-	-	-	-	-
	MRC-5	-	-	-	-	-	-	-
Po15/t	Vero	S	S	S	S	S	S	R
	B95a	-	-	-	-	-	-	-
	BCL	-	-	-	-	-	-	-
	CEF	-	-	-	-	-	-	-
	HeLa	-	-	-	-	-	-	-
	MRC-5	-	-	-	-	-	-	-

[1] = Vero-grown parental viruses
[2] S = Sensitive to MAb (no plaques observed)
[3] R = Resistant to MAb (plaques observed)
[4] - = Identical antigenic profile to the Vero-grown parental virus
[5] * = Resistant to MAb but smaller plaques observed

passages in HeLa cells (Table 4.3) and in the presence of MAbs 1721 and 1726 smaller plaques were formed, compared to in their absence.

Ur PT1 (sensitive to MAbs 1641, 1648, 1721 and 1726) became resistant to these MAbs after five passages in HeLa cells (Table 4.3). In addition, HeLa-derived virus formed smaller plaques in the presence of MAb 1641 compared to in its absence. No differences in antigenic profiles were observed between Ur PT3 and its various cell-derived virus progeny (Table 4.3).

B95a- and HeLa-derived Enders viruses were resistant to MAb 1689 in comparison to the sensitivity shown by the Vero-grown parental virus (Table 4.3). Rubini (sensitive to Mab 1641 and 1721) became resistant to these after five passages in B95a cells.

Viruses, derived by passage of Po15/t in different host cells, have identical antigenic profiles to the Vero-grown parental virus.

No changes in antigenic profile were detected for BCL, CEF or MRC-5 derived viruses, supporting the previous suggestion that no productive replication of MuV occurred during passage in these cells (see the section entitled, 'Growth of MuV in BCL, CEF or MRC-5 cells' in Chapter 3).

4.4 DISCUSSION

Seven MuV strains have been passaged five times in five different cell lines to investigate the effect of host cell passage on MuV. In this chapter, the virus progeny derived from these five passages have been investigated by comparison of plaque morphology and antigenic profile to determine whether host cell passage has generated phenotypic variants of their parents.

Table 4.4 summarises the results of Chapter IV and shows changes in plaque morphology were observed for two of the MuVs after passage in B95a cells (JL-2 and Rubini) and for two of the MuVs after passage in HeLa cells (Ur PT3 and Enders). Changes in antigenic profile were observed for three of the MuVs after passage in B95a cells (JL-2, Enders and Rubini) and for three of the MuVs after passage in HeLa cells (JL-5, Ur PT1 and Enders).

Plaque morphology may be influenced by several factors that affect viral replication and any difference in one of these factors could lead to alteration of plaque morphology. Such factors include the rate at which MuV attaches to and buds effectively from the host cell and the rate at which fusion of the viral and host cell membranes occurs. The HN protein is responsible for virus attachment and budding and the F protein is implicated in virus and cell fusion. A difference in these proteins could decrease or increase the rate at which virus enters and replicates in the host cell. Neuraminidase activity is also likely to affect plaque morphology, with a higher level leading to a quicker virus release rate and hence a larger plaque morphology because the released virus progeny infect the surrounding cells. Therefore, the neuraminidase activities of the different parental MuVs and of parental viruses and their respective cell-derived virus progeny, where plaque morphology changes are observed, might differ. It is also possible that other virus-encoded proteins are involved, for example, a change in the matrix (M) protein could lead to inefficient packaging and budding of mature virions.

Table 4.4: Summary of the results of Chapter IV.

VIRUS	CELL	PLAQUE SIZE[2]	ANTIGENIC PROFILE[4]
JL-2	Vero[1]	Clear, 1mm	1648 (S)
	B95a	0.5mm	R
	BCL	=[3]	=
	CEF	=	=
	HeLa	=	=
	MRC-5	=	=
JL-5	Vero	Clear, 1.5mm	1648 (S)
	B95a	=	=
	BCL	=	=
	CEF	=	=
	HeLa	=	R
	MRC-5	=	=
Ur PT1	Vero	Clear, 1mm	1641 (S),1648 (S), 1721(S), 1726 (S)
	B95a	=	=
	BCL	=	=
	CEF	=	=
	HeLa	=	R
	MRC-5	=	=
Ur PT3	Vero	Cloudy 0.5mm	NA
	B95a	=	=
	BCL	=	=
	CEF	=	=
	HeLa	1mm	=
	MRC-5	=	=
Enders	Vero	Clear, 0.5mm	1689 (S)
	B95a	=	R
	BCL	=	=
	CEF	=	=
	HeLa	<0.5mm	R
	MRC-5	=	=
Rubini	Vero	Cloudy, 0.5mm	1641 (S), 1721 (S)
	B95a	Clear	R
	BCL	=	=
	CEF	=	=
	HeLa	=	=
	MRC-5	=	=
Po15/t	Vero	Clear, 1mm	NA
	B95a	=	=
	BCL	=	=
	CEF	=	=
	HeLa	=	=
	MRC-5	=	=

1 Vero-grown parental virus
2 Plaque diameter
3 = No change from parental
4 Presented are the MAbs for which changes were observed between parental and progeny viruses. S = sensitive; R = resistant
NA = Not applicable

A plaque morphology difference can, therefore, be due to a multitude of factors; a change in any part of the viral replication cycle, from genome replication to protein synthesis to changes in cellular factors such as protein synthesis of cellular components necessary for viral replication.

Previously published results have shown that after passage of the wild type Matti strain of MuV in cells derived from normal human joint tissue, the viruses produced formed cloudy plaques in Vero cells when compared to the original parent (Huppertz and Chantler, 1991). Hence, it appears that passage of MuV in different host cells could affect virus replication and therefore plaque formation.

The results of this chapter indicate that passage of some MuVs in B95a or HeLa cells can produce progeny viruses with changes in their plaque morphology (Table 4.2). No consistent relationship is observed between the cell line from which virus was derived and a change in plaque morphology. The differences observed in plaque morphology are likely due to the differing ability of the viruses to infect Vero cells, after passage in B95a or HeLa cells and possibly due to host cell interactions.

There were some differences in the antigenic profiles of MuV determined in this study compared to a previous study (Yates *et al.*, 1996). Results from the study by Yates *et al* (1996) indicated that JL-2 and JL-5 were sensitive to 1641, rather than resistant, as was observed in this study and that the Urabe vaccine virus was sensitive to 1637, rather than resistant, as was observed for Ur PT1 and Ur PT3 in this study. The antigenic profiles of Enders and Rubini were identical between the two studies and the antigenic profile of Po15/t was not investigated by Yates *et al* (1996). It is possible that the differences were due to the different level of passage of the Vero-grown parental viruses used but differences could also have been detected due to technical error in either study. The plaque purification of Ur PT1 and Ur PT3 from the Urabe vaccine involved extra passages in Vero

cells and the JL-2 and JL-5 viruses used in this study have been passaged one extra time in Vero cells compared to the JL-2 and JL-5 used in the study by Yates *et al* (1996).

Neutralisation of virus results when antibody binds to a virus preventing replication and generally occurs by inhibition of virus binding to the cell receptor. In comparing the antigenic profiles of different cell-derived viruses with the parental (Vero-grown) virus, B95a-derived JL-2, HeLa-derived JL-5, HeLa-derived Ur PT1, B95a-derived Enders, HeLa-derived Enders and B95a-derived Rubini are different (Table 4.3). Some alternate-cell-derived viruses formed smaller plaques in neutralisation assays when compared to the Vero-derived control viruses. This suggested that there was some inhibition of replication by the MAb, insufficient to neutralise the virus completely.

The MAbs used in this project were raised against the Urabe strain of MuV and were reactive against the HN protein (Table 4.1; Yates *et al.*, 1996). Therefore, differences in antigenic profile between parent and progeny were assumed to be due to a difference in a particular antibody-binding site within the HN protein. JL-2, JL-5, Ur PT1 and Rubini progeny, after passage in either B95a or HeLa cells, showed resistance to MAbs 1641 and/or 1648. These MAbs are reactive against the neuraminidase active site (Table 4.1) and resistance to these MAbs indicates a possible genotypic change in the neuraminidase active site, within the HN protein. The neutralisation titre of MAb 1641 is low (Table 4.1) and hence virus resistance, by Ur PT1 and Rubini progeny, to this MAb is likely to be due to breakthrough virus and therefore, does not indicate a significant change in the neuraminidase active site. Breakthrough virus, or virus which has escaped neutralisation, can occur when the neutralisation titre of the antibody is low and the titre of the virus is high, therefore the amount of virus present is to high to be fully neutralised by the amount of antibody present.

Ur PT1, Enders and Rubini progeny, after passage in B95a or HeLa cells, showed resistance to MAbs 1689, 1721 and/ or 1726. These MAbs are reactive against the

haemagglutinin active site (Table 4.1) and resistance to these MAbs indicates a possible genotypic change within the active site for haemagglutination.

4.4.1 CONCLUSION

Results from this chapter indicate that passage of some MuVs in B95a or HeLa cells produces progeny virus with altered phenotypic characteristics. The production of altered phenotypes appears to be a chance association rather than dependant on the virus or the cell line because no correlation was detected between cell line, plaque morphology or antigenic profile. Investigation into whether these phenotypic changes correspond to genotypic changes, by sequence analysis of the HN and F proteins, was deemed necessary.

CHAPTER V

GENOTYPIC CHARACTERISATION OF THE HN PROTEIN

5.1 INTRODUCTION

Results of Chapters III and IV indicate the production of high quality MuV after passage in B95a or HeLa cells. Some of the viruses were plaque morphology and/or antigenic variants of their respective Vero-grown parental virus. Plaque morphology is affected by the rate of virus replication, possibly due to alterations in the virus attachment glycoprotein (HN). This protein is also the one against which the monoclonal antibodies used in this project are reactive. The 3D structure of the HN protein has not been deduced, and the structure of the receptor-binding pocket and the NA active site remains unidentified.

The aim of this chapter is to analyse the nucleotide and deduced amino acid sequences of the HN gene of Vero-grown parental viruses and their respective progeny after passage in 5 different cell lines in a comparison to determine whether genotypic differences were present. A second aim of this chapter was to compare the HN amino acid sequence of the Enders and Rubini strains.

5.2 EXPERIMENTAL PROCEDURES

The nucleotide sequence of the HN gene of the mumps vaccine virus strains JL-2, JL-5, Ur PT1, Ur PT3, Enders, Rubini and the wild type strain Po15/t was determined. Their respective amino acid sequences were deduced using start codon sequences of the standard triplet genetic code (Appendix I). To investigate the presence of genetic variants, the nucleotide sequences of the HN gene of MuVs after passages two and five in each different cell line were also determined, the respective amino acid sequences deduced and compared to that of their respective Vero-grown parent.

To analyse the nucleotide sequence of the HN gene, viral RNA was extracted from infectious tissue culture fluid, cDNA synthesized and the DNA amplified by PCR, as described in Chapter II. For each virus, the HN gene was amplified in three overlapping fragments (Figure 5.1A). Primers SH-7 and HN-2 were used to amplify the 5' fragment of

the HN gene. The middle fragment was amplified using HN-3 and HN-5 and the 3' fragment of the HN gene was amplified using HN-7 and L-19 (Figure 5.1B). The PCR products were purified for use in sequencing reactions performed using the aforementioned primers giving complementary data, one of forward sense and the second of reverse sense, and the products analysed using an ABI Prism 310 Genetic Analyzer. The resultant chromatograms were analyzed using the computer programs Chromas and GCG (Wisconsin Package Version 9.1).

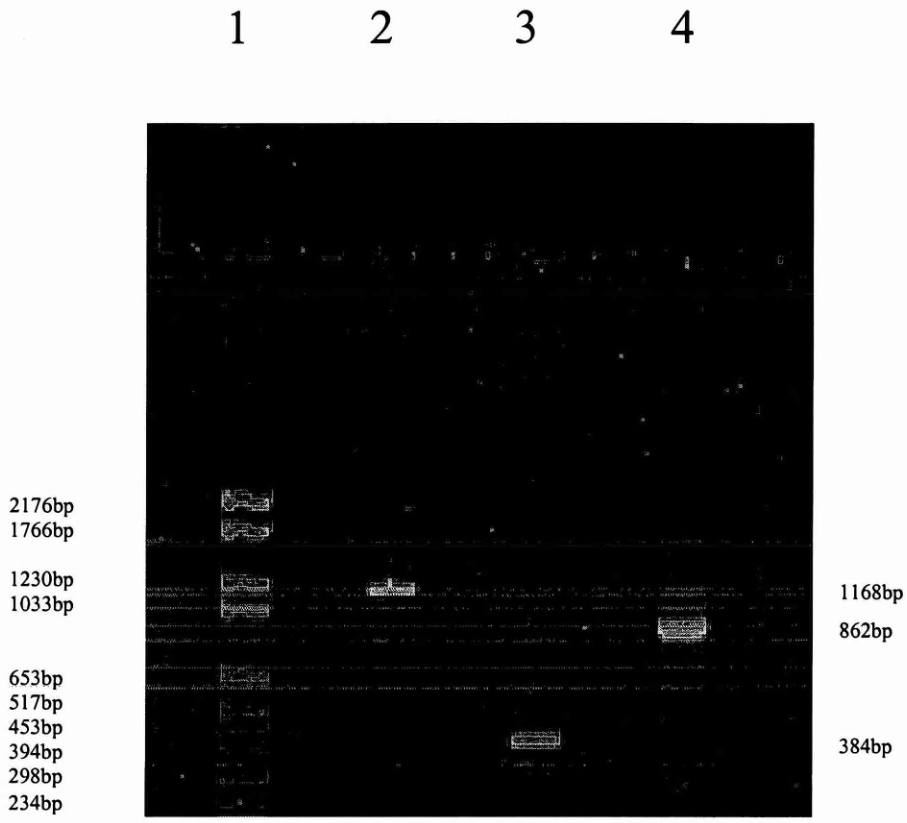
Any nucleotide differences observed between a progeny virus and a parental virus were confirmed by repetition of the sequence analysis.

5.3 RESULTS

5.3.1 MOLECULAR CHARACTERISATION of the MuV HN PROTEIN

The HN nucleotide and deduced amino acid sequence has previously been determined for JL-2, JL-5, Urabe (vaccine), Enders and Rubini (Yates *et al.*, 1996). Nucleotide sequence analysis of the HN gene of these viruses was repeated (Appendix II) in this project because the Vero-grown parental viruses were at higher passage level to those used in the study by Yates *et al* (1996). JL-2, JL-5 and Enders were passaged once more in this study, Ur PT1 and Ur PT3 had been passaged three further times and Rubini was passaged five further times in Vero cells, in comparison to the viruses analysed by Yates *et al* (1996). The Rubini analysed by Yates *et al* (1996) was unpassaged commercial vaccine. Some discrepancies were observed between data being derived in this project and the published data (Table 5.1), possibly due to the different passage levels of the viruses analysed. It is believed that the data determined in this project is accurate because of the numerous repetitions performed, the accuracy of previous data could be questioned. Ur PT1 and Ur PT3 analysed in this study were at the same passage level to the viruses analysed by Afzal *et al* (1998) but had been plaque purified three times in Vero cells in relation to the CEF-derived Urabe analysed by Yates *et al* (1996).

Figure 5.1A: Agarose gel electrophoresis of the PCR products after amplification of the HN gene in three overlapping fragments. Primer sequences are listed in Chapter II.



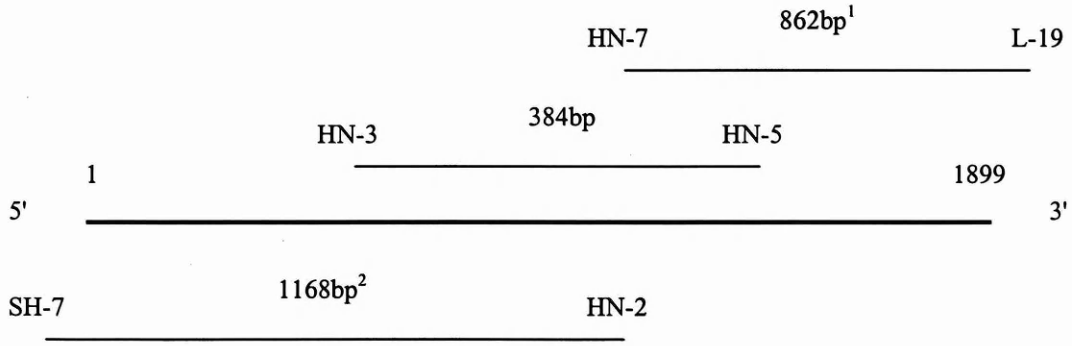
Lane 1: Molecular weight markers

Lane 2: PCR product amplified using primers SH-7 and HN-2 (1168bp)

Lane 3: PCR product amplified using primers HN-3 and HN-5 (384 bp)

Lane 4: PCR product amplified using primers HN-7 and L-19 (862 bp)

Figure 5.1B: Schematic diagram showing the positions of the primers, within mRNA sense RNA, used for amplifying and sequencing the HN gene.



[1] 111bp of L gene and 751bp of HN gene
[2] 109 of SH gene and 1059bp of HN gene

Table 5.1: The differences in the HN gene between previously published data (Yates *et al.*, 1996) and data determined in this project.

Virus	Nucleotide	Yates *et al*	This project	Amino acid	Yates *et al*	This project
JL-2	692	G	G/A	205	Arg	Arg/His
	719	C	T	214	Met	Met
	1733	T	C	552	Ala	Ala
JL-5	209	C	T	44	Thr	Ile
	1587	C	T	503	Gly	Gly
Urabe[1]	343[2]	A	G	89	Met	Val
	1081[3]	G	A	335	Glu	Lys
	1727[3]	A	G	550	Asp	Gly
Enders	96	C	A	6	Leu	Leu
	795	T	G	239	Leu	Leu
	1587	C	T	503	Gly	Gly
Rubini	96	C	A	6	Leu	Leu
	332	C	A	85	Ala	Asp
	460	T	C	128	Cys	Arg
	552	C	T	159	Ile	Ile
	795	T	G	239	Leu	Leu
	1587	C	T	503	Gly	Gly
	1611	T	C	511	Thr	Thr

[1] The HN gene of Urabe was analysed by Yates et al (1996). In this project the HN gene of Ur PT1 and Ur PT3 were analysed.
[2] This difference was only observed in Ur PT3.
[3] These differences were observed in Ur PT1 and Ur PT3.

The nucleotide sequence of the HN gene of Po15/t has not previously been determined and so no comparison could be made. The nucleotide and deduced amino acid sequences of the Po15/t HN gene have been submitted to the EMBL genetic database (accession number AJ238210).

Figure 5.2 shows the deduced amino acid sequence of the seven parental MuVs used in this study and includes a consensus sequence, obtained via comparison of the seven amino acid sequences; the amino acid residues in the consensus sequence are the most frequently observed at a particular position. The coding region of the HN gene is 1734 nucleotides in length and codes for 582 amino acids. The HN protein of the seven MuVs contains 7 conserved potential N-linked glycosylation sites (underlined in Figure 5.2). It is not known which, if any, are glycosylated but it is presumed that all are except one. The exception is considered to be the site located at residues 514-516 (N-P-T) because it contains a proline residue as the X amino acid (N-X-T/S), which is considered to inhibit glycosylation, possibly because of its structure. The HN protein also contains two heptad repeat regions; both located between amino acids 93 to 124, which can form coiled coil structures of α-helices. The amino acid sequence comprising these heptad repeat regions is conserved between the MuVs in this project, with the exception of 3 residues in Ur PT1, Ur PT3 and Po15/t, 1 in Enders, 2 in Rubini.

Figure 5.2: The deduced amino acid sequence of the HN protein from the seven MuVs analysed in this project. Potential N-linked glycosylation sites are underlined.

```
            1                                                        50
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ---I------
Ur PT1      -------T-- ---------F I---D----- ---------- ----------
Ur PT3      -------T-- ---------F I---D----- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15t       -------TI- ---------A-I---D----- ---------- ----------
CONSENSUS   MEPSKLFIMS DNATFAPGPV VNAAGKKTFR TCFRILVLSV QAVTLILVIV

            51                                                       100
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- V--------- ----------
Ur PT1      -----V---- ---------- -A-------T ---------- ----------
Ur PT3      -----V---- ---------- -A-------T --------V- ----------
ENDERS      ---------- ---------- ---------- V--------- ----------
RUBINI      ---------- ---------- ---------- V---D----- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
CONSENSUS   TLGELIRMIN DQGLSNQLSS ITDKIRESAA MIASAVGVMN QVIHGVTVSL

            101                                                      150
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ----I----- ----------
Ur PT1      ---------- ---------- GKK------- ---------- ----------
Ur PT3      ---------- ---------- GKK------- ---------- ----------
ENDERS      -----R---- ---------- ---------- ---------- ----------
RUBINI      -----R---- ---------- -------R-- ---------- ----------
Po15/t      ---------- ---------- SKK------- ---------- ----------
CONSENSUS   PLQIEGNQNQ LLSTLATICT NRNQVSNCST NIPLVNDLRF INGINKFIIE

            151                                                      200
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- ---------- ---------- ----------
Ur PT3      ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
CONSENSUS   DYATHDFSIG HPLNMPSFIP TATSPNGCTR IPSFSLGKTH WCYTHNVINA

            201                                                      250
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- -------A-- ---------- ---------- ----------
Ur PT1      ---------- -I-------- ---------- ---------- ----------
Ur PT3      ---------- -I-------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ------V---
CONSENSUS   NCKDHTSSNQ YVSMGILVQT ASGYPMFKTL KIQYLSDGLN RKSCSIATVP

            251                                                      300
JL-2        ---------- ---------- ---------- -------K-- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- ---------- ------V--- ----T-----
Ur PT3      ---------- ---------- ---------- ------V--- ----T-----
ENDERS      ---------- -----A---- ---------- ---------- ----------
RUBINI      ---------- -----A---- ---------- ---------- ----------
Po15/t      ---------- -----E---- ---------- ------V--- ----------
CONSENSUS   DGCAMYCYVS TQLETDDYAG SSPPTQKLTL LFYNDTITER TISPSGLEGN
```

Figure 5.2 continued:

```
            301                                                  350
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- -----L---- ----------
Ur PT1      ---------- ---------- ---------- ---------- ----------
UR PT3      ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- -----L---- ----------
CONSENSUS   WATLVPGVGS GIYFENKLIF PAYGGVLPNS TLGVKSAREF FRPVNPYNPC

            351                                                  400
JL-2        ---P------ ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      -----D---- ---------- -N-------- ---------- ----------
Ur PT3      -----D---- ---------- -N-------- ---------- ----------
ENDERS      ---P------ ---------- ---------- ---------- ----------
RUBINI      ---P------ ---------- ---------- ---------- ----------
Po15/t      L----D---- ---------- -N-------- ----S----- ----------
CONSENSUS   SGPQQELDQR ALRSYFPSTF SSRRVQSAFL VCAWNQILVT NCELVVPSNN

            401                                                  450
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- -Y--------
Ur PT1      ---------- ---------- ---------- ---------- ----------
Ur PT3      ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
CONSENSUS   QTLMGAEGRV LLINNRLLYY QRSTSWWPYE LLYEISFTFT NSGQSSVNMS

            451                                                  500
JL-2        ---------- ---------I ---------- ---------- ----------
JL-5        ---------- ---------- --I------- ---------- ----------
Ur PT1      ---------- -S---S---- ---A------ ---------- ----------
Ur PT3      ---------- -S---S---- ---A------ ---------- ----------
ENDERS      ---------- ---------- ------T--- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ------I---
Po15/t      ---------- ---------M ---A------ ---------- ----------
CONSENSUS   WIPIYSFTRP GLGNCRGENV CPTVCVSGVY LDPWPLTPYS HQSGINRNFY

            501                                                  550
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- ---------- --N------- ----------
Ur PT3      ---------- ---------- ---------- --N------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
CONSENSUS   FTGALLNSST TRVNPTLYVS ALNNLKVLAP YGTQGLFASY TTTTCFQDTG

            551                                    583
JL-2        ---------- ---------- ---------- --*
JL-5        ---------- ---------- ---------- --*
Ur PT1      ---------- ---------- ------T--- --*
Ur PT3      ---------- ---------- ------T--- --*
ENDERS      ---------- ---------- ---------- --*
RUBINI      ---------- ---------- ---------- --*
Po15/t      ---------- ---------- ------T--- --*
CONSENSUS   DASVYCVYIM ELASNIVGEF QILPVLARLT IT*
```

The HN gene is highly conserved between the related MuV strains sequenced in this study (Table 5.2).

TABLE 5.2: Percentage differences in the HN gene and protein sequences between seven parental MuV strains sequenced. The bottom left of the table depicts percentage differences in nucleotide sequence and the top right of the table depicts percentage differences in amino acid sequence.

Virus	JL-2	JL-5	Ur PT1	Ur PT3	Enders	Rubini	Po15/t
JL-2		1.72	3.95	4.12	1.03	1.37	3.61
JL-5	3.11		4.64	4.81	1.89	2.06	3.95
Ur PT1	7.06	7.90		0.17	4.30	4.64	2.92
Ur PT3	7.11	7.95	0.05		4.47	4.81	3.09
Enders	1.53	2.37	6.64	6.69		0.69	3.95
Rubini	1.63	2.47	6.69	6.74	0.29		4.30
Po15/t	7.42	7.85	4.58	4.63	6.74	6.74	

5.3.2 MOLECULAR COMPARISON of the ENDERS and RUBINI HN GENE

Rubini is considered to have arisen by a laboratory contamination with Enders and it was of interest to compare their HN gene nucleotide sequence to investigate if this theory can be substantiated. Comparison of the previously published HN nucleotide sequence of Enders and Rubini (Yates *et al.*, 1996) revealed three differences between the two strains, coding for two amino acid differences at positions 477 and 497 (Table 5.3). Comparison of the HN nucleotide sequences, determined in this project, indicate five differences between the two strains (Tables 5.2 and 5.3), coding for four amino acid differences at position 85, 128, 477, 497.

Table 5.3: The differences between the HN genes of Enders and Rubini.

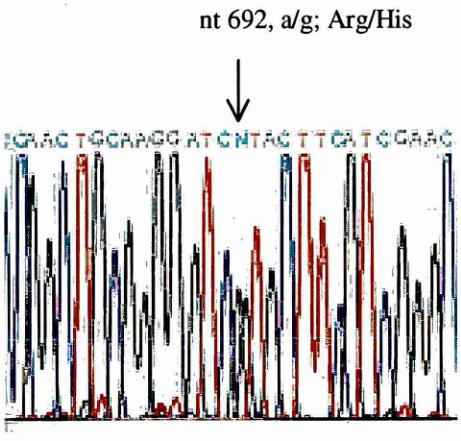
Nucleotide / amino acid	Yates *et al.*, 1996		This project	
	Enders	Rubini	Enders	Rubini
332 / 85	C / Ala	C / Ala	C / Ala	A / Asp
460 / 128	T / Cys	T / Cys	T / Cys	C / Arg
1507 / 477	A / Thr	T / Ser	A / Thr	T / Ser
1558 / 497	G / Arg	T / Ile	G / Arg	T / Ile
1611 / 511	C / Thr	T / Thr	C / Thr	C / Thr

5.3.3 EFFECT of HOST CELL PASSAGE on MuV GENOTYPE

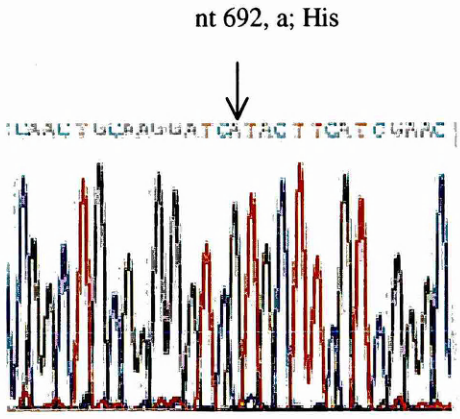
The nucleotide sequences of the HN gene of MuVs were determined after the second and fifth passages in each cell line and together with the deduced amino acid sequence, were compared with the respective sequences of the HN gene and its protein of the Vero-grown parental viruses. Original data showing coding nucleotide sequence differences are presented in Figure 5.3 and the results are summarised in Table 5.4. Nucleotide differences were detected for all of the viruses after passage in at least one cell line with one exception, Po15/t (no differences were detected between this Vero-grown parental virus and its progeny derived from passage in any of the cell lines). Where differences in nucleotide sequence were detected, the sequence analysis was repeated to confirm the results.

Figure 5.3: Chromatograms, depicting the amino acid substitutions between parental viruses and their progeny in the HN protein. A) JL-2, B) Ur PT1, C) Ur PT3, D) Enders and E) Rubini.

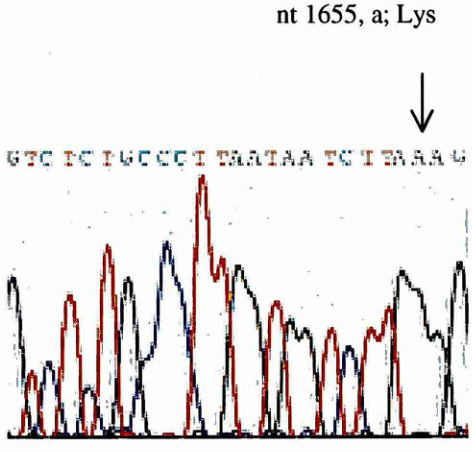
A) Vero-grown parental JL-2



JL-2 passaged on B95a cells



B) Vero-grown parental Ur PT1



Ur PT1 passaged on HeLa cells

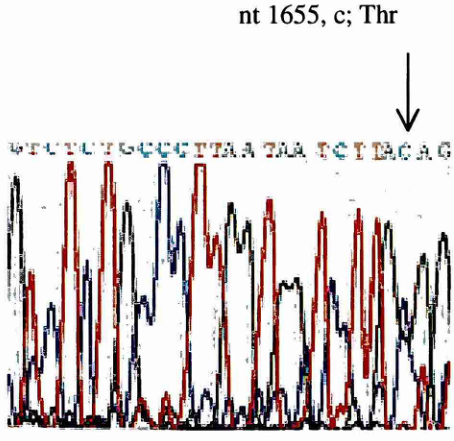
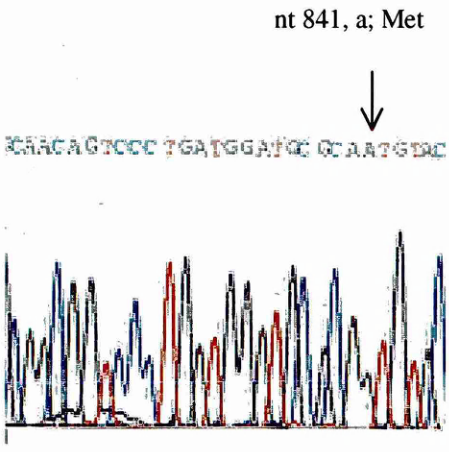


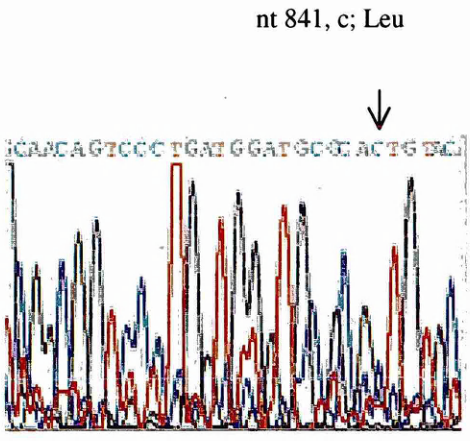
Figure 5.3 continued:

C) Ur PT3

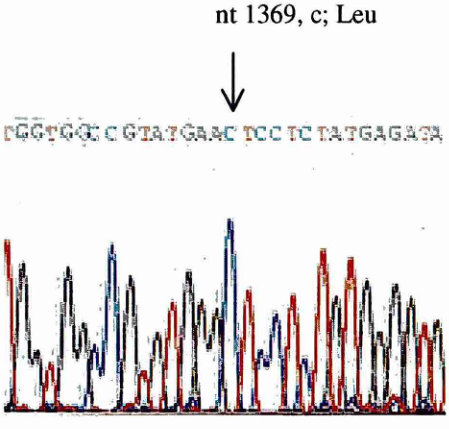
Vero grown parental Ur PT3



Ur PT3 passaged on HeLa cells



Vero grown parental Ur PT3



Ur PT3 passaged on MRC-5 cells

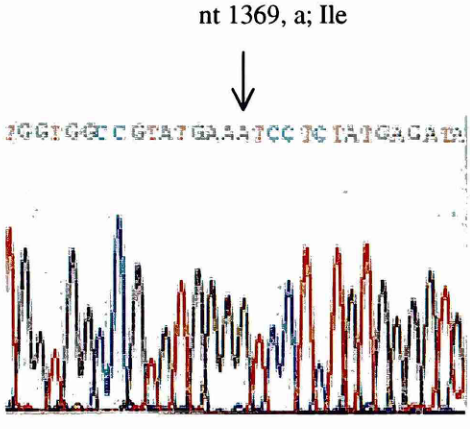
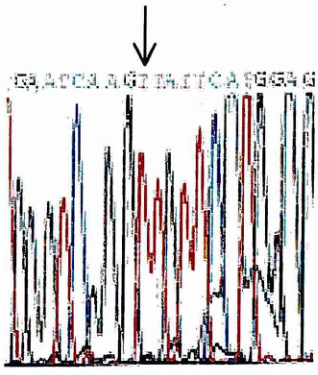


Figure 5.3 continued:

D) Enders

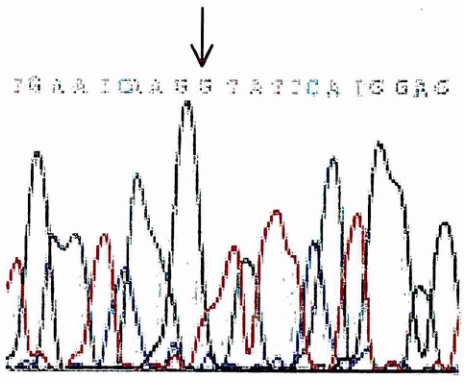
Vero-grown parental Enders

nt 353, t; Val



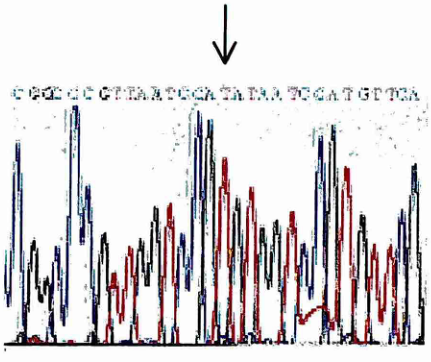
Enders passaged on B95a cells

nt 353, g; Gly



Vero-grown parental Enders

nt 1117, t; Tyr



Enders passaged on HeLa cells

nt 1117, g; Asn

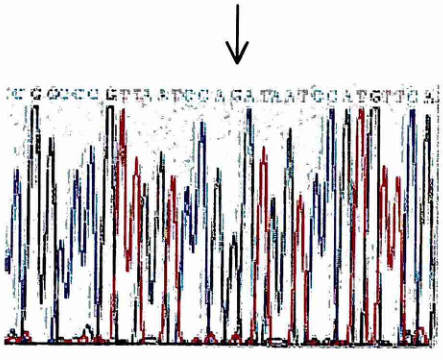
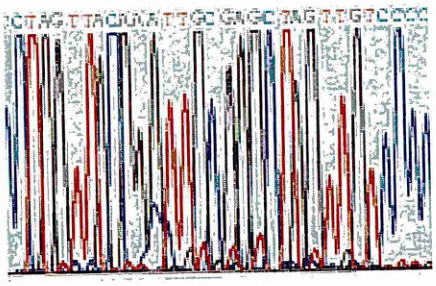


Figure 5.3 continued:

E) Vero-grown parental Rubini

nt 1252, t; Cys



Rubini passaged on B95a cells

nt 1252, c; Arg

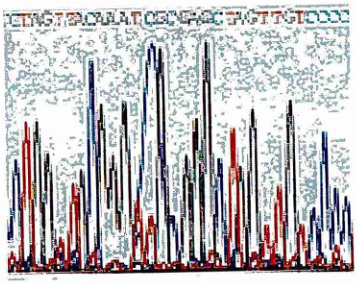


Table 5.4: Nucleotide and deduced amino acid substitutions detected in the HN gene of progeny viruses after passage in different host cells.

Virus	Cell line	Passage level	Nucleotide Difference	Amino acid Difference
JL-2	B95a	2 and 5	692, A/G → A	205, R/H → H
	B95a	2 and 5	729, T → C	Silent
	HeLa	2 and 5	729, T → C	Silent
	B95a + Vero[1]	2 and 4	692, A/G → A	205, R/H → H
	B95a + Vero[1]	2 and 4	729, T → C	Silent
JL-5	B95a	2 and 5	1515, T → C	Silent
Ur PT1	B95a	5	1269, C → T	Silent
	HeLa	5	1655, A → C	526, K → T
Ur PT3	HeLa	5	841, A → C	255, M → L
	MRC-5	5	1369, C → A	431, L → I
Enders	B95a	2 and 5	353, T → G	92 V, → G
	HeLa	2 and 5	1117, T → G	347 Y, → N
Rubini	B95a	5	1252, T → C	392 C, → R

[1] JL-2 derived from two passages in B95a cells was passaged in Vero cells four times to investigate reversion.

Summary

- B95a-derived JL-2, Enders and Rubini are genetic variants, when compared to their Vero-grown parental viruses.
- HeLa-derived Ur PT1, Ur PT3 and Enders virus progeny are genetic variants, when compared to their Vero-grown parental viruses.
- No genotypic differences in the HN gene were detected between Po15/t and its cell-derived progeny.
- MRC-5-derived Ur PT3 is a genetic variant, when compared to its Vero-grown parental virus.
- Silent mutations were observed in viruses derived from B95a or HeLa cells.
- No genotypic differences were detected between BCL or CEF-derived viruses and their Vero-grown parental viruses.

5.4 DISCUSSION

The HN protein is involved in virus attachment to cells and also partly in effective virus budding. Sequence analysis of the HN gene was performed for viruses passaged in a variety of different host cells to investigate its involvement in host cell adaptation. Where differences in the molecular structure of the HN protein were detected, sequence analysis was repeated to confirm the result.

Initially nucleotide analysis of the HN gene was performed for the parental viruses. The percentage difference of nucleotide and amino acid residues within the HN protein of the strains sequenced is relatively low (Table 5.2) when compared to the SH gene. The SH gene is highly variable between strains (Afzal *et al.*, 1997, Wu *et al.*, 1998), showing 2.3% to 13.4% nucleotide sequence variation between the isolates analysed and the consensus sequence (Afzal *et al.*, 1997). The HN genes of the MuVs analysed in this study show upto 7.95% nucleotide sequence variation.

The data presented here indicate that passage of some MuVs in certain cell lines (B95a, HeLa and MRC-5) produces viruses with genotypic differences when compared to their Vero-grown parents. Genetic changes were observed in the form of nucleotide differences, several of which resulted in amino acid changes at residues 92, 205, 255, 347, 392, 431 and 526. In addition to coding differences, silent substitutions were also detected. Silent differences arise due to random mutation during virus replication but it is also possible but unlikely that a nucleotide difference could have been introduced, in error, by the Taq polymerase used in the amplification of DNA from viral cDNA prior to sequencing.

Nucleotide analysis of the JL-2 HN gene indicated cross-banding between two nucleotides at position 692 (A/G, coding for amino acids His/Arg at residue 205 (Figure 5.3A)), suggesting that the Vero-grown parental virus is a mixed population. After the second passage in B95a cells this cross-banding was not observed; nucleotide A was detected (coding for amino acid His) but not nucleotide G (Figure 5.3A). Nucleotide 692 was a G in the data determined by Yates *et al* (1996) (Table 5.1). Therefore, it appears that the parental population has evolved to adapt to growth in its various environments; changing at amino acid 205 from an Arg, in virus passaged twice in Vero cells (Yates *et al.*, 1996) to Arg/His, in virus passaged three times in Vero cells (JL-2 parental virus used in this project) to a His, in virus passaged in B95a cells. No reversion is observed in the HN gene of B95a-derived virus after further passage in Vero cells.

The Enders and Rubini strains of MuV have been observed to be genetically closely related, with few differences previously detected in their HN gene (Yates *et al.*, 1996). Sequence data, determined in this project, support previous results that Enders and Rubini are very closely related to one another and, therefore, previous suggestions that one of these viruses arose as a laboratory contaminant of the other appear possible. The Enders and Rubini strains sequenced in this project had different passage histories, which could account for the few amino acid differences. Passage of these two strains in the same cell

substrate did not select for the same mutations. This either suggests that, for Enders and Rubini to grow in B95a or HeLa cells, these amino acid differences were not necessary or were necessary to compensate for the differences already observed between the two strains.

Passage of MuV in Vero cell lines had previously been observed to select for genetic variants with amino acid substitutions in their HN protein at positions 335 and 464 (Yates, 1995). It called into question the suitability of Vero cells for the propagation and assaying of MuV because certain viruses after passage in Vero cells were different from the original vaccine virus.

The MuVs, which contain amino acid differences in their HN gene, possibly contain differences in HN protein structure and/or function, enabling them to propagate on the B95a, HeLa or MRC-5 cell line to a greater extent than their Vero-grown parental virus. However, the observed mutations could be random mutations with no deleterious effect and mutations, responsible for adaptation, could be present elsewhere in the genome.

Viruses identified in this study with genetic differences were probably present in the original Vero-grown virus population but at a level too low to be detected by sequence analysis. The viruses, containing the amino acid differences, then outgrew the Vero-grown parental viruses and sequence differences were detected.

To complement the data determined in this chapter, where only a few of the progeny viruses contained changes in their HN genes when compared to the Vero-grown parents, analysis of the nucleotide sequence of the second glycoprotein, the F protein, was performed. It was also of interest to sequence the F gene of Enders and Rubini to observe the similarity between the two strains.

CHAPTER VI

GENOTYPIC CHARACTERISATION OF THE F PROTEIN

6.1 INTRODUCTION

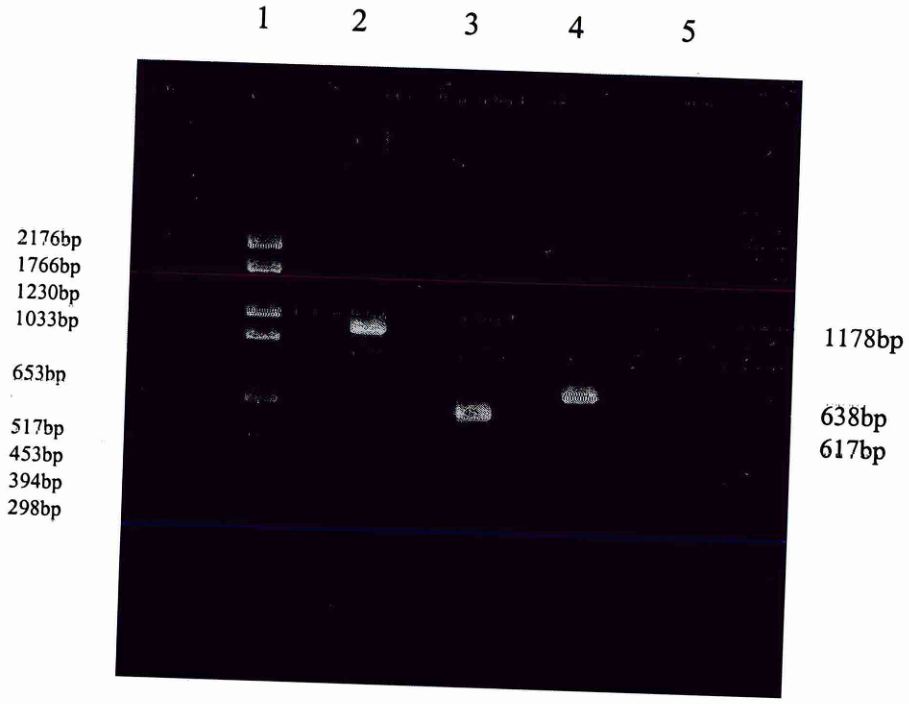
Results from the previous three chapters indicate that growth of MuV in B95a or HeLa cells produces progeny of improved fitness. Some of the progeny are plaque morphology and/or antigenic variants of their parent and nucleotide differences within some of their HN genes have been detected, which could account for the observed phenotypic differences. It was also of interest to analyse the nucleotide sequence of the F gene of the progeny viruses to determine if any of the phenotypic variants were also genetic variants with respect to their F gene. In addition, this would extend available information on the MuV F protein structure, providing more information about its function.

6.2 EXPERIMENTAL PROCEDURES

The nucleotide sequence of the F gene of the Vero-derived parental viruses JL-2, JL-5, Ur PT1, Ur PT3, Enders, Rubini and Po15/t was determined. Their respective amino acid sequences were deduced using the start codon of the standard triplet genetic code (Appendix I). The nucleotide sequence of the F gene of MuVs after passages two and five in each different cell line was also determined and the respective amino acid sequences deduced. The nucleotide and amino acid sequence of the F gene from the progeny viruses were then compared with that of their respective parental virus to determine the presence of genetic variants.

To analyse the nucleotide sequence of the F gene, viral RNA was extracted from infectious tissue culture fluid, cDNA synthesized and the DNA amplified by PCR as described in Chapter II. For each virus, the F gene was amplified in three overlapping fragments. Primers M-4 and F-4 were used to amplify the 5' fragment of the F gene. The middle fragment was amplified using F-10 and F-12 and the 3' fragment of the F protein gene was amplified using F-6 and F-2 (Figure 6.1B). Figure 6.1A shows gel electrophoresis of the three DNA fragments after amplification. The PCR products were purified for use in

Figure 6.1A: Agarose gel electrophoresis of the three overlapping fragments of DNA after PCR amplification of the MuV F gene.



Lane 1: Molecular weight markers of following base pairs: 2176bp, 1766bp, 1230bp, 1033bp, 653bp, 517bp, 453bp, 394bp and 298bp.

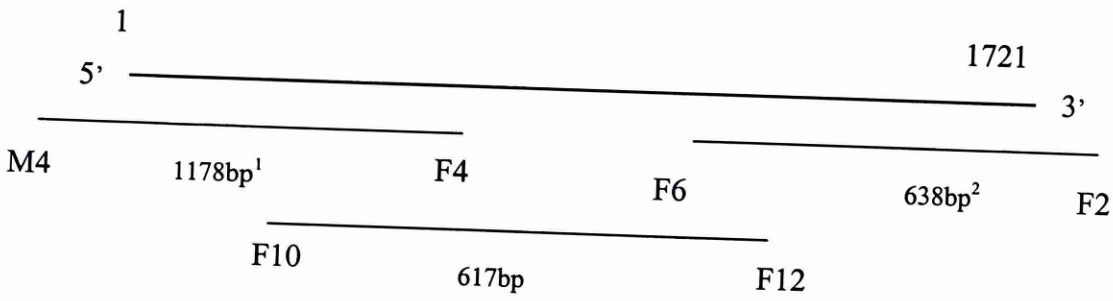
Lane 2: Product of PCR amplification using primers M-4 and F-4 (1178 base pairs).

Lane 3: Product of PCR amplification using primers F-10 and F-12 (617 base pairs).

Lane 4: Product of PCR amplification using primers F-6 and F-2 (638 base pairs).

Lane 5: Negative control.

Figure 6.1B: Schematic diagram showing the positions of the primers, within mRNA sense RNA, used for amplifying and sequencing the F gene.



[1] 430bp of M gene and 748bp of F gene
[2] 589bp of F gene

sequencing reactions, which were performed using the aforementioned primers giving complementary data, and the resultant chromatograms were analyzed as described in Chapter IV.

6.3 RESULTS

6.3.1 MOLECULAR CHARACTERISATION of the MuV F PROTEIN

The nucleotide and deduced amino acid sequences of the JL-2, JL-5, Enders, Rubini and Po15/t F proteins were determined as described above and in Chapter II and have been submitted to the EMBL genetic database (accession numbers AJ010821, AJ133694, AJ133694, AJ009685 and AJ010300 respectively). The nucleotide and deduced amino acid sequences of Ur PT1 and Ur PT3, although determined, were not submitted because Ur Am9, the original Urabe vaccine strain, was submitted previously (Cusi *et al.*, 1995).

The F gene sequence contains 1721 nucleotides (Appendix III) with the first start codon located at nucleotide position 64. The coding region was 1614 nucleotides in length, from nucleotide 64 to 1677 inclusive (the first stop codon was from 1678 to 1680), coding for 538 amino acids. Figure 6.2 shows the deduced amino acid sequence of the virus strains analysed and also includes the amino acid sequence for SBL-1, RW and Ur Am9 viruses and a consensus sequence, obtained via comparison of the amino acid sequences; the amino acid residues used in the consensus sequence are the most frequently observed at a particular position.. The inactive form of the F protein (F0) is cleaved into two subunits to form the fusogenic form, which consists of two subunits, F2, from amino acid 1 to 102 and F1, from amino acid 103 to 538 inclusive (436 amino acids in length). The signal peptide at position 1 to 19 is underlined and shows high variation amongst the strains sequenced; this high variation indicates the signal sequence is not a major determinant of infectivity. Potential N-linked glycosylation sites are depicted in dark blue. All the F proteins of the virus strains sequenced in this study contain a potential N-linked glycosylation site in the

FIGURE 6.2: Deduced amino-acid sequence of the F protein of MuVs. Possible N-linked glycosylation sites are in blue, the cysteine residues are in green, the F protein cleavage site is shown in red, the fusion peptide is shown in light blue, the signal sequence is underlined and the transmembrane domain is shown in pink. The figure includes a consensus sequence and the sequences of the SBL-1 (Elango *et al.*, 1989), RW (Waxham *et al.*, 1987) and UrAm9 (Cusi *et al.*, 1995) strains of MuV.

```
            1                                                  50
JL-2        ---Y------ ---------- ---------- ---------- ----------
JL-5        -N-------- Y--------- ---T------ ---------- ----------
UrPT1       ----L-T--S -V----V--- ---------- ---------- --------I-
UrPT3       ----L-T--S -V----V--- ---------- ---------- --------I-
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ----S-T--S -V-------- ---------- ---------- --------I-
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ----S----- --V------- ---------- ---------- --------I-
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   MKAFPVICLG FAIFSSSICV NINILQQIGY IKQQVRQLSY YSQSSSSYVV

            51                                                 100
JL-2        ---------- ---------I ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----L-----
UrPT1       ---------- ---------- ---------- ---------- ----S-----
UrPT3       ---------- ---------- ---------- ---------- ----S-----
ENDERS      ---------- ---------- ---------- ---------- T---------
RUBINI      ---------- ---------- ---------- ---------- T---------
Po15/t      ---------- -D-------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- T---------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------I ---------- ---------- ---------- ----S-----
Consensus   VKLLPNIQPT DNSCEFKSVT QYNKTLSNLL LPIAENINNI ASPSPGSRRH

            101                                                150
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   KRFAGIAIGI AALGVATAAQ VTAAVSLVQA QTNARAIAAM KNSIQATNRA

            151                                                200
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ------S--- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----F-----
UrPT3       ---------- ---------- ---------- ---------- ----F-----
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          I--------- ---------- ---------- ---------- ----Y-----
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   VFEVKEGTQQ LAIAVQAIQD HINTIMNTQL NNMSCQILDN QLATSLGLYL
```

Figure 6.2 continued:

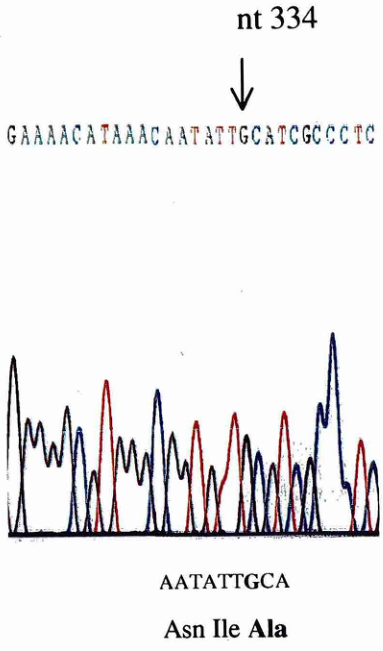
```
            201                                                  250
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- -----T----
UrPT3       ---------- ---------- ---------- ---------- -----T----
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -T-------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- -----T----
Consensus   TELTTVFQPQ LINPALSPIS IQALRSLLGS MTPAVVQATL STSISAAEIL

            251                                                  300
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------I ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   SAGLMEGQIV SVLLDEMQMI VKINIPTIVT QSNALVIDFY SISSFINNQE

            301                                                  350
JL-2        ---------- ---------- -----P---- ---------- ----------
JL-5        ---------- ---------- ---------- M--------- ----------
UrPT1       ---------- -------S-- ---------- ---------- ----S-----
UrPT3       ---------- -------S-- ---------- ---------- ----S-----
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -------S-- ---------- ---------- ----S-----
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- -------S-- ---------- ---------- ----S-----
UrAm9       ---------- -------S-- ---------- ---------- ----S-----
Consensus   SIIQLPDRIL EIGNEQWRYP AKNCKLTRHH IFCQYNEAER LSLETKLCLA

            351                                                  400
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------T ---------- --------N- ----------
UrPT3       ---------- ---------T ---------- --------N- ----------
ENDERS      ---------S ---------- ---------- ---------- ----------
RUBINI      ---------S ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
SBL-1       ---------S ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------T ---------- --------N- ----------
Consensus   GNISACVFSP IAGSYMRRFV ALDGTIVANC RSLTCLCKSP SYPIYQPDHH
```

Figure 6.2 continued:

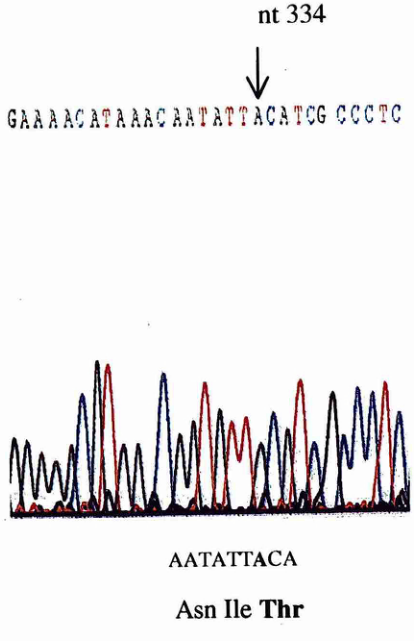
```
            401                                                  450
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       --------A- ---------- ---------- ---------- ----------
UrPT3       --------A- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- --T-------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      --------A- ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          --------T- ---------- ---------- ---------- ----------
UrAm9       --------A- ---------- ---------- ---------- ----------
Consensus   AVTTIDLTSC QTLSLDGLDF SIVSLSNITY AENLTISLSQ TINTQPIDIS

            451                                                  500
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ------F--G --------S- ----------
UrPT1       ---------- ---------- ---------N ---------- ----------
UrPT3       ---------- ---------- ---------N ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------N --------S- ---------L
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------N ---------- ----------
UrAm9       ---------- ---------- ---------N ---------- ----------
Consensus   TELSKVNASL QNAVKYIKES NHQLQSVSVS SKIGAIIVAA LVLSILSIII

            501                                       538
JL-2        ---------- ---------- ---------- --------*
JL-5        ---------- ---------- ---------- --------*
UrPT1       ---------- ---------- ---------- --------*
UrPT3       ---------- ---------- ---------- --------*
ENDERS      ---------- ---------- ---------- --G-----*
RUBINI      ---------- ---------- ---------- --------*
Po15/t      -I-------- ---------- -------L-- --------*
SBL-1       ---------- ---------- ---------- --------*
RW          ---------- ---------- ---------- --------*
UrAm9       ---------- ---------- ---------- --------*
Consensus   SLLFCCWAYI ATKEIRRINF KTNHINTISS SVDDLIRY*
```

Figure 6.3: Chromatograms obtained by nucleotide sequencing of the F gene depicting the nucleotide difference in both Enders and Rubini, which translates to an amino acid substitution resulting in the addition of an extra possible N-linked glycosylation site.

a) JL-2, JL-5, Ur PT1, Ur PT3 and Po15/t



b) Enders and Rubini



F2 subunit at residues 73 to 75 (NKT); however, the Enders, Rubini and SBL-1 strains contain an additional site at residues 89-91 (NIT). Figure 6.4 shows an example of a chromatogram, obtained from nucleotide sequencing of the F gene at the site of the extra N-linked glycosylation site. In the F1 subunit, all strains contain five potential N-linked glycosylation sites at amino acid residues 182-184 (NMS), 352-354 (NIS), 427-429 (NIT), 433-435 (NLT) and 457-459 (NAS).

The cleavage site within the F protein between F2 and F1, between amino acids 102 and 103, is highlighted in red and underlined and consists of the amino acid sequence Arg-Arg-His-Lys-Arg (Waxham *et al.*, 1987). A protease is responsible for cleavage immediately after this sequence, between residues 102 and 103, forming a mature fusogenic protein with a fusion peptide at the N-terminus of F1 consisting of residues 103 to 128, depicted in light blue (Figure 6.2). This domain is completely conserved amongst the MuVs sequenced to date. Adjacent to the fusion peptide is heptad repeat A (HRA), from position 130 to 178. A cysteine rich region, containing eight conserved cysteine residues (depicted in green), is located from amino acid 324 to 410. Heptad repeat B (HRB) is located between positions 453 and 477. The transmembrane region is underlined and highlighted in pink at positions 485-509. Conservation of the residues within HRA is observed for all seven mumps virus strains sequenced except for JL-5, which contained a Ser at position 177 (position f within HRA) instead of an Asn. Conservation of HRB was observed except for JL-5, which had a Phe at position 477 (position d in HRB) instead of a Val as observed in all other F proteins sequenced.

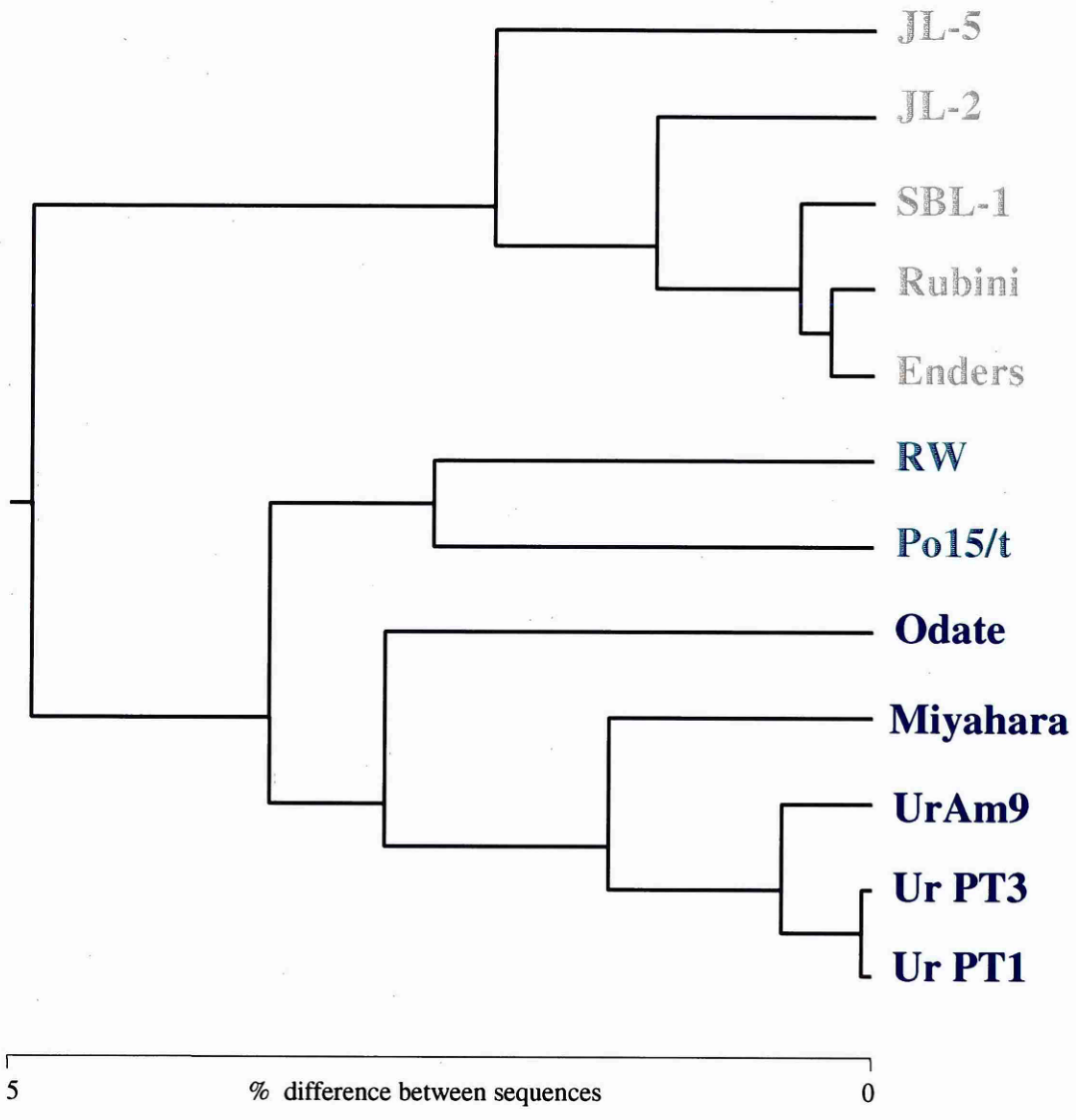
The percentage difference of nucleotide and amino acid residues between the F gene and protein of the strains sequenced is relatively low (Table 6.1). Percentage nucleotide differences were calculated as a percentage of the whole gene, including non-coding regions. The most variable region of the F protein is the signal sequence (Figure 6.2).

TABLE 6.1: Percentage differences in the F protein between the seven MuV strains sequenced. The bottom left of the table depicts percentage differences in nucleotide sequence and the top right of the table depicts percentage differences in amino acid sequence. Values were rounded up to 2 decimal places.

VIRUS	JL-2	JL-5	PT1	PT3	ENDERS	RUBINI	Po15/t
JL-2		2.23	3.34	3.34	1.30	0.74	3.15
JL-5	3.00		4.08	4.08	2.41	2.04	3.71
PT1	5.64	5.93		0.00	3.53	3.15	2.23
PT3	5.58	5.87	0.58		3.53	3.15	2.23
ENDERS	1.53	2.44	5.23	5.17		0.37	3.15
RUBINI	1.39	2.21	5.29	5.23	0.35		2.97
Po15/t	5.52	5.70	3.78	3.72	5.17	5.17	

The relationship between the nucleotide sequences was illustrated by constructing a tree diagram using a distance matrix approach (Figure 6.5). This is intended to show groupings of similar sequences and is not intended to imply any evolutionary relationship. In this sense, it is strictly a phenogram (Nei *et al.*, 1987). The distance between each pair of aligned sequences was calculated as the number of mismatched nucleotide bases, as a percentage of the sequence length and does not include nucleotides from the non-coding region. All sequences covered identical regions, and there were no gaps. The sequence alignment and calculation of distances was performed using the GCG software package (Wisconsin Package Version 9.1). The sequences were grouped using the average distance method (UPGMA) (Wisconsin Package Version 9.1) using an in-house program based on the SAS statistical package clustering procedure (SAS/STAT Users Guide, 1989). The phenogram includes data for the SBL-1, RW, Odate, Miyahara and Ur Am9 strains of MuVs. The relationship between the nucleotide sequence of the F gene of MuVs determined in this project and of other MuV strains that had previously been sequenced shows three clear groupings of MuVs (Figure 6.5). JL-2, JL-5, Enders and Rubini were grouped together with SBL-1. Ur PT1 and Ur PT3 were grouped together with the Ur

Figure 6.4: Phenogram showing the relationship between the nucleotide sequences of the F genes of different MuV strains.



Am9 strain, the Miyahara strain and the Odate strain. The wild-type Po15/t was grouped separately with the RW strain.

The F proteins of JL-2 and of JL-5 differ by ten amino acids (2.23%), which are located in the signal peptide at positions 2, 4 and 11 and at positions 24, 70, 95, 177, 326, 331, 477, 480 and 489. The two Urabe strains, Ur PT1 and Ur PT3 are grouped together and their F genes appear the closest, with a percentage nucleotide difference of 0.58 and no amino acid differences, showing full conservation of the secondary structure of the F protein between these two MuV strains. Enders and Rubini are also very closely related with two amino acid differences (0.37%) in the F protein, which are at position 443, a Thr in Enders and an Asn in Rubini and the second at position 533, a Gly in Enders and an Asp in Rubini. The wild type isolate included in this study, Po15/t, was isolated from the throat of a patient with mumps in Portugal in 1996 in Vero cells. The F gene of Po15/t has a percentage nucleotide difference of between 3.72 to 5.70% (Table 6.1) when compared to the F genes of the other MuVs sequenced and a 2.23 to 3.71% difference in the deduced amino acid sequence (Table 6.1). The F protein of Po15/t is observed to be more closely related to the F proteins of the two Urabe viruses than the Jeryl Lynn, Enders or Rubini strains (Table 6.1). This wild type virus has a conserved fusion peptide domain and other structurally important amino acids, such as cysteines and prolines, are also conserved. If percentage amino acid differences are calculated omitting the signal peptide sequence, the values are lower (considerably in some cases) but the pattern of relationship between the viruses remains the same.

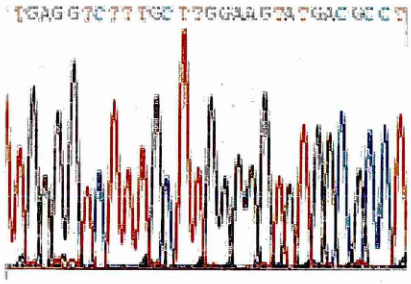
6.3.2 EFFECT of HOST CELL PASSAGE on MuV GENOTYPE

After two and five passages in the various different cell lines, the F gene of the seven different strains was sequenced and compared with that of the parental Vero-grown viral sequence. No differences were observed between any virus passaged in any cell line, when compared to the Vero-grown parental virus, except for JL-2. The F gene of JL-2, derived by five passages in B95a cells, contained a difference at nucleotide 751 (A → G), which coded for an amino acid change at position 230 (Ser → Gly) (Figure 6.5). No other nucleotide and hence amino acid substitutions were observed in the F gene of virus derived from passage in different cell lines.

Figure 6.5: The chromatograms showing the results of nucleotide analysis of the F gene; the mutation between JL-2 and its B95a-derived progeny is shown.

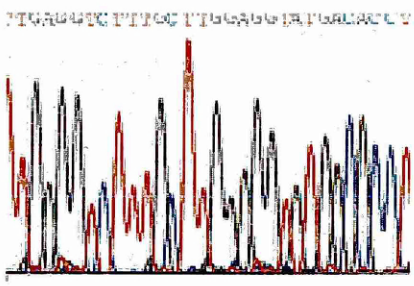
JL-2

nt 751, a; Ser



JL-2 passaged on B95a cells

nt 751, g; Gly



6.4 DISCUSSION

6.4.1 F PROTEIN STRUCTURE

The fusion gene of seven MuV strains was sequenced. The length of the nucleotide and deduced amino acid sequences were identical to those reported for other MuV strains (Elango *et al.*, 1989; Waxham *et al.*, 1987; Takeuchi *et al.*, 1989; Cusi *et al.*, 1995) at 1721 nucleotides and 538 amino acids.

The amino acid sequence of the F gene shows high conservation with the exception of the signal sequence. Full conservation of the residues within HRA and HRB is observed with the exception of JL-5, which contained a Ser at position 177 (position f within HRA) instead of an Asn and a Phe at position 477 (position d in HRB) in place of a Val. These substituted amino acids retained the hydrophobicity of the HR regions. The F protein of Enders and Rubini contains an extra potential N-linked glycosylation site in the F2 peptide when compared to the other viruses studied. The SBL-1 strain of MuV also contains this extra potential N-linked glycosylation site and the phenogram (Figure 6.4) indicates that the F proteins of Enders, Rubini and SBL-1 are closely related. The glycosylation status of this or any other site is unknown but is highly likely because N-X-T/S sites are usually glycosylated, especially for virus glycoproteins.

Critical amino acids, such as cysteine, are important for the biological activity of proteins. Cysteine residues are important for structure and conformation and are also involved in linking F1 and F2, the two subunits of the mature F protein. The cysteine residues in the F protein are highly conserved. Other structurally important amino acids such as proline, glycine and alanine also show a high level of conservation.

The cytoplasmic domain of the F protein also appears important for syncytia formation, by comparison to NDV. Mutational analysis implicated a region of ten amino acids at position 540 to 550, important for syncytia formation by NDV (Sergel and Morrison, 1995). The cytoplasmic domains of the F protein of the MuV strains studied here show

high conservation of residues, implicating the importance of this region. It is possible that the cytoplasmic domain interacts with cellular proteins such as cytoskeletal components that participate in syncytia formation.

The phenogram, generated from the nucleotide sequence of the F gene indicates three groups. JL-2, JL-5, SBL-1, Enders and Rubini constitute one group, RW and Po15/t constitute a second group and Odate-1, Miyahara, Ur PT1, Ur PT3 and Ur Am9 constitute the third group. These results uphold a recent study of the relationship between the SH protein genes of mumps viruses (Wu *et al.*, 1998). The first group corresponds to group A, European and North American isolates, the second group corresponds to group D, also European and North American isolates and the third group corresponds to group B, Japanese isolates plus a few UK isolates. The percentage difference of nucleotide and amino acid residues within the F protein of the strains sequenced is relatively low (Table 6.1) when compared to other genes, such as the SH gene. The SH gene is highly variable between strains (Afzal *et al.*, 1997; Wu *et al.*, 1998), where upto 13.4% nucleotide differences are detected between strains (Afzal *et al.*, 1997). The phenogram showing the relationship between the various MuV strains, with respect to their F protein, shows comparable grouping to a phenogram presented by Yates *et al* (1996), with respect to the HN protein of MuVs. The high level of conservation between the F genes suggests that the function of this protein is important and that there is little room for variability.

The F protein of the MuV strains included in this study contain two heptad repeat regions that are highly conserved and contain hydrophobic residues at their a and d positions (Figure 6.6). The MuV protein sequences described here are consistent with the structures reported for HIV and influenza fusion proteins, gp41 and HA respectively.

6.4.2 EFFECT of HOST CELL SELECTION on the GENETIC PROPERTIES of MuV.

The F protein was completely conserved between viruses derived after passage in five alternate cell lines and their Vero-grown parental strain except for JL-2, passaged five times in B95a cells, where a nucleotide difference at position 751 (A → G) resulted in a coding difference (Ser → Gly).

A previous study selected genetic variants of MuV by passage, which has altered structure and pathogenicity. Borskin *et al* (1992) observed that the Leningrad-3 strain of MuV, when passaged in QEF cells, after either 8 or 38 passages, differed at nucleotide 501 in the F protein, an A residue after 8 passages and a G residue after 38 passages. The virus passaged 8 times was also observed to contain several regions of cross banding, suggesting that this virus consisted of more than one variant. The authors found no change in the P gene and did not examine the HN or any other viral gene.

6.4.3 CONCLUSION

The high conservation of the F protein between MuVs suggests that the function of this protein is completely defined and the role it performs does not allow for much change in primary or secondary structure and the selection of only one F protein variant from all the MuVs passaged on all the different cell lines confirms this. The MuV F protein resembles the F protein of other paramyxoviruses and of other RNA viruses suggesting that the role it performs and the method it uses to perform these roles is similar.

Figure 6.6: Amino acid sequence of the two HR regions located within the F1 subunit of the F protein of the seven MuV strains analysed in this study. Underlined residues were not completely conserved between the strains sequenced. Includes sequences for other paramyxoviruses: MV (Richarson *et al.*, 1986), NDV (Chambers *et al.*, 1986) and Sendai Virus (Blumberg *et al.*, 1985). Residues at positions a and d are capitalised and highlighted. The conservation of hydrophobic amino acids within these HR regions is clear.

A) HRA

MuV AqtNaraIaaMknsIqaTnraVfeVkegTqqLaiaVqaIqdhIntImntQ

MV SmlNsqaIdnLrasLetTnqaIeaGqemIlaVqgvQdyInneLipSmnqL

NDV AkqNaanIlrLkesIaaTneaVheVtdgLsqLavaVgkMqqfVndQfnkT

SeV AreAkrdIalIkesMtkThksVelLqnaVgeQilaLktLqdfVndEikpA

B) HRB (leucine zipper motif)

MuV LskVnasLqnAvkyIkeSnhqLqsVsvs

MV LgnAiakLedAkelLesSdqiLrsMkgl

NDV LgnVnnsIsnAldkLeeSnskLdkVnvk

SeV LadAtnfLqdSkaeLekArkiLseVgrw

CHAPTER VII

GENERAL DISCUSSION

7.1 INTRODUCTION

Tissue culture is an invaluable tool in the study of MuV infection because, to date, no optimal animal model has been identified. Vero cells are currently used for isolation, propagation and assaying of MuVs; however, the appropriateness of this is under question after a previous study concluded that growth of MuV in Vero cells produces virus populations that are different from the original isolate (Yates., 1995). Yates (1995) passaged MuVs on Vero and CEF cells, and observed that variants were selected with a different antigenic pattern compared to the previous, predominant population. The Vero adapted Urabe vaccine strain of MuV was neutralised by a panel of anti-HN MAbs, whereas the commercial bulk Urabe vaccine, adapted to CEF cells, was not neutralised by one of the MAbs. Sequencing of the HN gene revealed one amino acid difference between the two at position 335. In the CEF isolate, no selection occurred and the amino acid at 335 was a Lys. In the Vero adapted virus, selection occurred and the amino acid at 335 was a Glu. The author also observed that passage in Vero cells appeared to select either a virus with a Lys, instead of an Asn, at position 464 or a Glu, instead of a Lys, at position 335, but not both. The study by Yates (1995) called into question the suitability of using Vero cells for MuV propagation and especially for wild type MuV isolation from patients.

During the period of study leading to this thesis, several cell lines were investigated to increase our understanding of the mechanisms involved in host cell passage of MuV and to move towards an optimum host cell with which to study MuV replication *in vitro*. This thesis describes the phenotypic and genotypic analysis of MuV after passage in a variety of host cells by the application of virological and molecular techniques. Tables 7.1A and B provide a brief summary of the results of passaging MuVs in B95a or HeLa cells and indicate increased viral infectivity, with occasional phenotype or genotype alterations in comparison to the Vero-adapted parental virus.

Table 7.1A: Summary of the differences observed between Vero-grown viruses and their B95a-derived progeny.

Virus	Fitness	Plaque morphology	Antigenic profile	HN	F
JL-2	+[1]	✓[2]	✓	205 (R→H)	230 (S→G)
JL-5	-/+[3]			S[4]	
Ur PT1	+			S	
Ur PT3	+				
Enders	+		✓	92 (V→G)	
Rubini	+	✓	✓	392 (C→R)	
Po15/t	+				

Table 7.2: Summary of the differences observed between Vero-grown viruses and their HeLa-derived progeny.

Virus	Fitness	Plaque morphology	Antigenic profile	HN	F
JL-2	-/+				
JL-5	-/+		✓		
Ur PT1	-/+		✓	526 (K→T)	
Ur PT3	-/+	✓		255 (M→L)	
Enders	-/+	✓	✓	347 (Y→N)	
Rubini	-/+				
Po15/t	-/+				

[1] +, Increase in virus fitness without prior adaptation period.

[2] ✓, Difference in phenotype detected.

[3] -/+, Increase in virus fitness after a period of adaptation.

[4] S, Silent change detected

In addition to Vero cells, two other cell lines have been identified as possible alternatives for the analysis of mumps infection *in vitro*, B95a and HeLa cells. The data suggest that, although the HN protein is important for virus attachment and entry to the cell, the observed phenotypic changes are not likely to correspond to the observed genotypic changes in the HN. The observed genotypic changes in the HN are likely to be coincidental. This is based on observations that, in the majority of cases, a difference in plaque morphology or antigenicity coincides with a difference in the molecular structure of the HN protein but no correlation is observed between those properties. For approximately half of the B95a and HeLa-derived viruses, no phenotypic or genotypic change was observed beyond an increase in viral fitness, again suggesting no correlation between phenotypic and genotypic changes as far as tested. After one or several passages of a virus under different environmental conditions the virus population may consist of a different proportion of variant to parent and hence the virus population may show an altered genotype and may also show altered phenotypic characteristics. This concept has previously been observed for MuV growth in Vero and CEF cells (Afzal *et al.*, 1998; Yates *et al.*, 1996) and has now been observed for some MuVs after growth in B95a, HeLa and MRC-5 cells.

7.2 EFFECT of HOST CELL PASSAGE on MuV PHENOTYPE and GENOTYPE

Viral fitness is defined as the ability of a virus to produce infectious progeny and was measured using the $TCID_{50}$ assay. HA and NA assays were performed to detected any virus activity not detected by the $TCID_{50}$ assay. In addition to replicating viruses, non-replicating viruses also contain HA and NA activity, hence these assays were not used to determine the infectivity of the MuVs. After passage in B95a cells, MuVs significantly increased in fitness without an apparent adaptation period, with the exception of one variant of the Jeryl Lynn vaccine (JL-5). After passage in HeLa cells, MuVs significantly increased in fitness following an apparent adaptation period. Adaptation to a cell line is

usually indicative of a difference in virus genotype because it is likely that a virus, which has increased its fitness for a particular cell substrate after a period of adaptation, will be a variant of the original Vero-derived parent (Section 1.12). It was thus anticipated that B95a-derived viruses would potentially be identical in genotype to the Vero-grown parent, with the exception of JL-5, whilst HeLa-derived viruses would much more likely be variants of their respective Vero-grown parent. However, where genotypic and other phenotypic differences were expected they were not identified in all cases and vice-versa.

In addition to increased fitness some, but not all, of the progeny viruses after passage in B95a or HeLa cells had an altered phenotype and genotype. Four progeny viruses had altered plaque morphologies, and three of these coincided with a change in antigenic profile; the exception was Ur PT3 (Tables 7.1 A and B). Three further viruses had changes in antigenic profile which did not coincide with a difference in plaque morphology (Tables 7.1 A and B). Six viruses had amino acid changes in their HN protein and each one coincided with altered plaque morphology and/or antigenicity. Only one virus had a change in the F protein. Seven of the virus progeny from passage in B95a or HeLa cells did not contain a phenotype or amino acid change, although two silent changes were detected. This was not unexpected for the B95a-derived viruses (except for JL-5) because there was no apparent period of adaptation but was unexpected for the HeLa-derived viruses because there was a clear period of adaptation.

These results imply that the role of the HN protein in cell tropism is ambiguous for the cell lines used in this study because sequence changes were not identified in all cases of adaptation, change in antigenicity or change in plaque morphology. For example, JL-5 provided the most dramatic evidence for adaptation of all the viruses during its growth in B95a cells, where infectivity was barely detectable for the first two passages but then increased significantly. Curiously, this dramatic adaptation event did not coincide with a

sequence difference in the HN or F proteins or any other phenotypic difference. Presumably other viral proteins are involved in host cell tropism.

Where differences in antigenicity were detected (in some viruses after passage in B95a or HeLa cells), they were anticipated to be due to substitutions within the HN protein sequence because the MAbs were raised against this protein and this was generally the case, with one exception (JL-5 passaged in HeLa cells). The substituted amino acid residues in the HN protein responsible for monoclonal antibody escape in the progeny viruses are:

- 205 (JL-2), 92 (Enders) and 392 (Rubini) after passage in B95a cells.
- 526 (Ur PT1) and 347 (Enders) after passage in HeLa cells.

No amino acid difference was detected which could explain the difference in antigenicity between JL-5 and its progeny after passage in HeLa cells; the resistance was likely due to breakthrough virus. There was no clear correlation between escape from neutralisation and a particular MAb or between escape from neutralisation by a specific MAb and substituted amino acid residues in the HN protein.

Differences in plaque morphology identified between parental Vero-grown viruses and their progeny also coincided with substitutions in the HN protein (Tables 7.1 A and B). However, no correlation was observed between the genotypic and phenotypic changes, therefore, the HN protein is not implicated as the cause of these phenotypic changes. The alterations in plaque morphology are likely due to differences between host cell factors present in the cell line used in growth of the virus and present in the cell line used for assaying the viruses (Vero cells).

The relative positions of the amino acid residues implicated in monoclonal antibody escape within the three-dimensional structure of the HN protein are unknown, as this has not been determined. However, using computer predictions kindly performed by Dr. Mark Forster, NIBSC, it was possible to predict the secondary structure of the HN protein, based on the

published sequence of the HN protein of JL-2 and using a computer program developed by the Protein Bioinformatics Group at the University of Warwick; the results are presented in Figure 7.1. The computer predictions are based on careful cross validation of related protein chains.

The predicted structure of the HN protein, presented in Figure 7.1, shows an initial random coil region of 5 amino acids at the N-terminal end of the protein, followed by a 4 amino acid region of strand. Following the strand region is another region of random coil, then an α-helical structure including residues 26 to 60, presumed to be the transmembrane domain. Alpha helices were also predicted between amino acid residues 71 to 118, presumed to include both heptad repeat regions. There are many predicted regions of 'strand', indicative of β-pleated sheets, and often two strands are separated by a short region of random coil, suggesting the β pleated sheets run in opposite direction to one another.

The mutations observed in this study (Tables 7.1A and B) are randomly located in different regions of the HN protein (Figure 7.1). Amino acids at position 205, 347 and 392 are located in regions of random coil, amino acid at position 92 is in an α-helix region and amino acids at positions 255 and 526 are located in strand regions. No differences in the were observed when the computer predictions were performed using the mutated sequences, suggesting the mutations had no effect on the structure of the HN protein.

Figure 7.1: The predicted structure of the HN protein. The degree of confidence was determined through comparison of known related sequences. C = random coil, E = strand and H = helix.

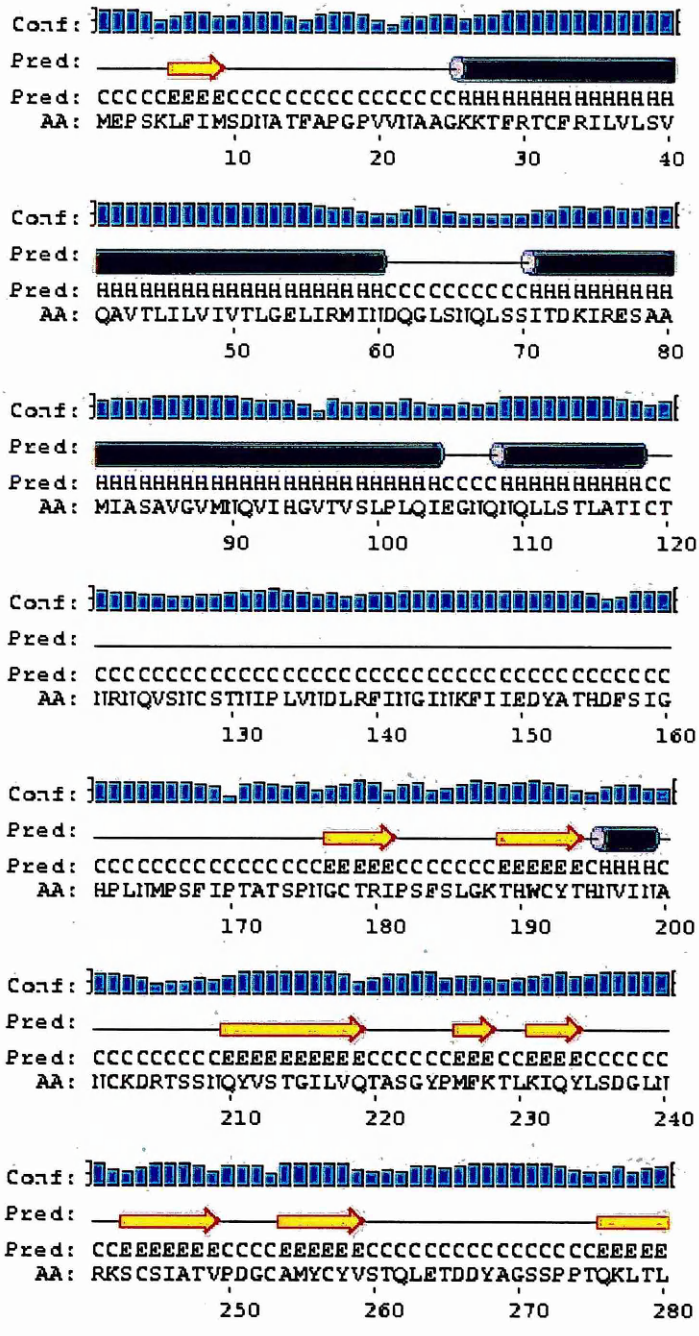


Figure 7.1 continued:

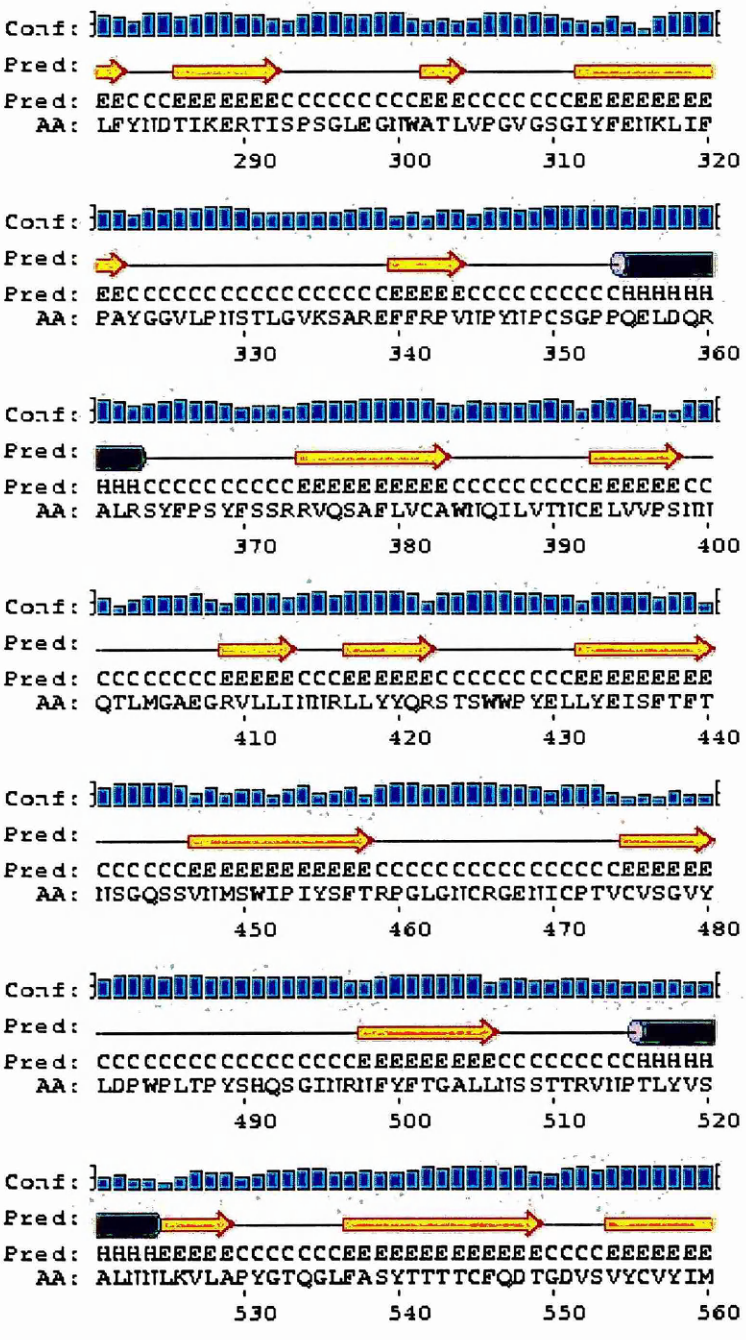
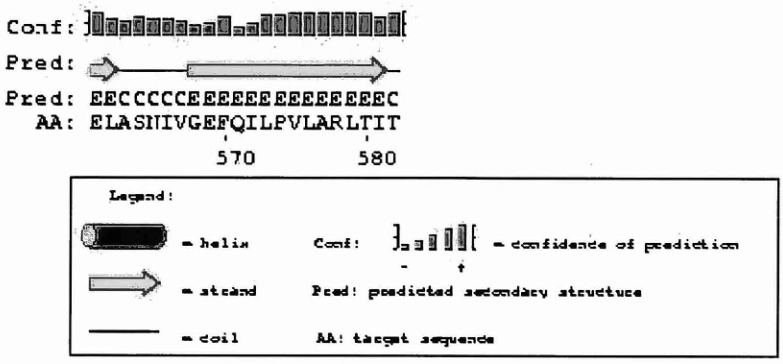


Figure 7.1 continued:



Data for predicting whether amino acids are buried or exposed within the HN protein was determined using an alternate programme (Rost *et al.*, 1995), data not shown. This programme predicts accessibility of amino acids by comparison of each amino acid in the protein sequence with the Dictionary of Secondary Structure of Proteins (DSSP). The results of this programme indicate that only two of the amino acids identified above are exposed (residues 205 and 347) and that all the other amino acids (92, 255, 392, 526) are buried within the HN protein (data not shown).

The two amino acids identified as being exposed (residues 205 and 347) are not implicated in the MAb resistance observed because resistance to the same MAbs in JL-5, Ur PT1 and Enders did not coincide with these mutations. It is possible that amino acids at positions 205 and 347 were partially responsible for the changes in plaque morphology observed in JL-2 and Enders.

A molecular difference within the HN protein of Ur PT3 after passage in MRC-5 cells was identified at amino acid residue 431 with no apparent effect on virus plaque morphology or antigenicity. This difference coincided with the apparent adaptation of this virus to MRC-5 cells. Using the program developed by Rost *et al* (1995), this amino acid is predicted to be buried within the HN protein, and therefore, is not implicated in the apparent adaptation to MRC-5 cells (data not shown).

MuV growth in BCL, CEF and MRC-5 (except for Ur PT3) cells resulted in no growth of virus. Virus activity detected by the $TCID_{50}$ assay was likely remaining input virus. Evidence of true replication could have been obtained by performing western blotting to assay for the viral proteins involved in replication. The low levels of infectious progeny could be due to several factors, including the inability of MuV to attach to the cell, to enter the cell and/or to replicate within the different environment. Also, for infectious virus to be produced, the mature F protein requires cleavage by an appropriate protease. Therefore, the distribution of protease in the host is also a critical factor in determining virus tropism.

Growth of MuVs in the presence of external trypsin was not attempted in this study but could have aided virus growth.

It appears that the two Urabe viruses were less affected by passage than the other viruses. They maintain a good titre in B95a cells with no changes in plaque morphology, antigenicity or protein sequence and in HeLa cells the required adaptation period for Ur PT1 and Ur PT3 was minimal compared with the other strains. Takeda *et al* (1998) showed that Vero-grown measles virus (MV) lost its ability to replicate and induce syncytia in B95a cells, and they implicated the P and L proteins. It appears that a similar effect has happened with the Urabe viruses because Vero-grown Ur PT1 and Ur PT3 caused less cell fusion in B95a cells in contrast to the other MuVs investigated. However, these two MuVs did replicate and produce infectious progeny upon infection of B95a cells. Unfortunately, no other virus proteins of MuVs were analysed in this study.

Host cell tropism and cell to cell spread of paramyxoviruses has been investigated in detail recently with different studies implicating different viral proteins as being responsible. A study by Yates (1995) implicated two amino acids in the adaptation of the Urabe strain of MuV to Vero or CEF cells, at positions 335 and 464 within the HN protein (section 1.12); the sequences of the other viral genes were not analysed. These amino acids were conserved between parental Vero-adapted viruses and their progeny in this study either suggesting that they were not important for determining host cell range but possibly for other phenotypic characteristics or that no adaptation occurred in this study.

Passage of wild type measles virus (MV) in Vero cells results in virus progeny that is attenuated (Kobune *et al.*, 1990), whilst the use of B95a cells isolates virus that closely resembles the wild type in that it remains infectious for humans. Takeda *et al* (1998) analysed and compared the genome sequences, glycoprotein structure and function, and gene expression and replication of a wild type MV isolated in B95a cells and its Vero-adapted counterpart upon growth in B95a cells. Genome differences were detected in the

H, P and L proteins. Further examination of the H proteins from the B95a or Vero-derived viruses showed that they were equally able to support fusion and it was concluded that the amino acid changes appeared to be co-incidental. Gene expression and replication was investigated by blocking *de novo* protein synthesis, amplifying the mRNAs obtained from primary transcription and confirming the specificity of each band by Southern blotting. The specific mRNA products after B95a infection with Vero-adapted virus were significantly less abundant than those from B95a-adapted virus; therefore, the genome replication of the Vero-adapted virus was significantly impaired in B95a cells compared with that of the B95a-adapted virus. The authors implicated the P and L proteins in this significantly impaired gene transcription and concluded that MV adaptation to growth in Vero cells results in transcriptional attenuation in B95a cells due to amino acid changes located in the polymerase and accessory proteins.

A study by Johnston *et al* (1999) also looked at host cell tropism of MV, this time by functionally analysing the syncytium-inducing ability of MV F and H proteins of vaccine and wild-type strains in different combinations in transfected Vero or HeLa cells. They concluded that the H and F proteins were necessary for MV host cell tropism but that other factors were also important. By mutational analysis, the M protein has also been shown to play an important role in MV spread (Cathomen *et al.*, 1997), by interacting with the cytoplasmic tails of the F and H proteins, inhibiting cell-to-cell fusion and promoting the assembly of virus particles.

Virus glycoproteins are often implicated in host cell tropism because they attach the virus to the cell. However, because of the ubiquitous nature of the MuV cellular receptor, sialic-acid located on glycoproteins and glycolipids, in this study it is likely that the HN protein is not implicated in host cell tropism. This could have been verified with adsorption studies. Relating the genotypic and phenotypic characteristics of viruses studied for this thesis and comparing them to the other studies mentioned, it seems likely that the envelope

glycoproteins, whilst highly implicated, do not solely determine MuV cell tropism or virus phenotype.

7.3 The HN PROTEIN

The HN protein is responsible for attaching the virus to the cellular receptor, a sialic-acid containing glycoprotein or glycolipid. It also interacts with the F protein to allow virus fusion with the host cell membrane. Data determined in this study suggests that molecular differences identified in the HN protein of the MuVs after passage in different host cells are coincidental and are not involved in determination of virus phenotype.

Unlike Ur PT3, Ur PT1 grown on HeLa cells, cannot haemagglutinate horse erythrocytes and these viruses differ by only one amino acid (89) in their HN protein. The region within the HN protein responsible for attachment to erythrocytes is unknown but potentially this amino acid forms part of the receptor-binding site. Using the program developed by Rost *et al* (1995) for predicting secondary structure and accessibility, this residue is located within an α-helical region but is buried within the helix and is therefore not implicated in this event (data not shown). It is unknown how this difference in the haemagglutinating ability occurs.

For influenza virus, neuraminidase activity is partly responsible for aiding in the budding of progeny viruses by preventing them reabsorbing to the infected cell and in the removal of sialic acid to prevent self-aggregation of virus particles (Palese *et al.*, 1974). It is assumed that the MuV neuraminidase has a similar role. Of the parental Vero-grown viruses, only Enders and Rubini possessed detectable neuraminidase activity. Amino acids within the HN protein of Enders and Rubini, which were identical to each other but different to the HN protein of the other MuVs, were residues 106 and 266. It is probable that one or both of these amino acids form part of the neuraminidase active site. Active sites for neuraminidase have been proposed previously to be located between amino acids

93 and 110 (Wang *et al.*, 1999) and between 241 to 246 (Jorgensen *et al.*, 1987). Amino acid 106, Arg in Enders and Rubini and Gly in the other viruses, is within one of these proposed neuraminidase active sites, suggesting that it can affect the NA activity of MuVs and is within an HR region, an important region for HN function, possibly both for neuraminidase activities and for interaction with the F protein (Stone-Hulslander *et al.*, 1999). Amino acid 266, Ala in Enders and Rubini and Asp in the other viruses, except Po15/t (Glu), possibly also affects the NA activity of mumps viruses, where an Ala increases activity and a Glu decreases activity compared to Asp (the neuraminidase activity of Po15/t was lower than other viruses). Using structural prediction programmes developed by Rost *et al* (1995), residues 106 and 266, determined in this project as being important for neuraminidase activity, are predicted to be exposed within the HN protein, compatible with their involvement in the neuraminidase active site (data not shown). Sites proposed previously to form neuraminidase active sites (residues 93 to 110 and 241 to 246) are predicted to be buried; the first is within an α-helical region and the second within a strand region.

Previous observations have suggested that levels of neuraminidase can affect the rate of fusion (Merz *et al.*, 1981) and hence the plaque morphology of a virus. MuV only produces low levels of neuraminidase and in this study, no correlation is observed between NA activity and fusion activity or plaque morphology.

Although results from this study do not solely implicate the HN protein in determining host cell tropism, studies on other viruses have suggested that envelope glycoproteins play an important role. Analysis of molecularly cloned isolates of HIV-1 with different tropism for immortalized CD4+ cell lines, has identified part of the V3 loop of one of the envelope glycoproteins, gp120, as the major determinant of cell tropism (Chavda *et al.*, 1994). Although having a different sequence, the V3 region of the viral envelope protein, SU, of feline immunodeficiency virus (FIV) is also implicated in host cell tropism, through

characterisation of chimeric infectious molecular clones containing envelope gene sequences (Vahlenkamp *et al.*, 1999).

The envelope glycoprotein of the rhabdovirus infectious hematopoietic necrosis virus (IHNV) has also been implicated in playing an integral part in tissue tropism (Kim *et al.*, 1994). Kim *et al* (1994) analysed the genomes of neutralisation resistant variants of IHNV that had been selected with a glycoprotein (G)-specific monoclonal antibody and compared the sequence with the parental strain. One of the variants was highly attenuated with markedly different tissue tropism and sequence analysis indicated that two changes detected in the G protein were responsible for the altered tissue tropism of the variants.

7.4 The F PROTEIN

The F protein is responsible for MuV fusion with cell membranes, during virus entry into the cell and during the creation of multinucleated cells or syncytia when an infected cell fuses with a non-infected cell. Cytopathic effect in an infected cell line is an indication of virus infectivity. Mumps viruses did not produce CPE in BCL, CEF or MRC-5 cells; even Ur PT3, the only MuV to adapt to MRC-5 cells, did not produce CPE. In contrast, in MuV-infected B95a and HeLa cells, CPE was observed. The CPE in MuV-infected B95a cells and HeLa cells differed between MuV strains, Ur PT1 and Ur PT3 causing less cell fusion than the other MuV strains; this did not seemingly affect the infectivity titres of theses two viruses in B95a or HeLa cells. The F proteins of the two Urabe viruses differ from all the other strains by seven amino acid substitutions at positions 5 (Pro → Leu, in the Urabe viruses), 17 (Ser → Val), 95 (Pro → Ser), 195 (Ser → Phe), 246 (Ala → Thr), 370 (Val → Thr) and 389 (Ser → Asn). The amino acid differences at positions 5 and 17 are located within the variable signal sequence of the F protein, and changes in this region are not considered important for the decreased cell fusion observed. Amino acid 95 is located within the F2 subunit just prior to the cleavage site. Amino acid 195 is located on

the C terminal side of HRA (Joshi *et al.*, 1998), and amino acids 370 and 389 are located to the N terminal side of HRB and within a cysteine rich area. Amino acids 95, 195, 246, 370 and 389, separately or together, probably affect the fusogenic properties of the MuV F protein. Mutational analysis of the RW F protein showed that replacement of Ser-195 by aromatic amino acids such as Trp or Phe, significantly reduced the fusion inducibility of an otherwise fusion competent F protein (Tanabayshi *et al.*, 1994). If this observation holds true for other strains of MuV, it could account for the reduced cell fusion caused by Ur PT1 and Ur PT3. The reduced ability to induce syncytium formation could also be due to differences in the P and L proteins, as discussed previously (Takeda *et al.*, 1998).

For some paramyxoviruses, including MuV, the F protein also requires the presence of the HN protein for efficient fusion. The HN protein contains two HR domains, between amino acids 93 and 125, which are thought to form α-helical structures (Figure 7.1), could possibly interact with the HR domains of the F protein (Stone-Hulslander *et al.*, 1999). Mutations in these HN HR domains adversely affected fusion promotion. The HN proteins of Ur PT1 and Ur PT3 contain mutations in the second HR domain when compared to the other MuVs. Mutations were observed at amino acid positions 121 to 123, NRN → GKK. It is possible that these mutations adversely affect the fusion capability by an altered interaction between the HN and F proteins of the two viruses. However, Po15/t also had mutations at the same site, NRN → SKK, but its fusion ability was not adversely affected. Therefore, if these residues in the HN protein are important for fusion activity, the double K residue potentially is not implicated in adverse affects on fusion whilst residue 121 could be, where a Gly reduces fusion ability. Residue 121 is located in an area of random coil (Figure 7.1) and is predicted to be exposed (Rost *et al.*, 1995), implying that it could interact with the F protein.

The F genes of the seven MuV strains under study were sequenced and a high conservation level was observed except for within the signal peptide. The signal peptide contains a

sequence which not only directs and anchors the F protein in the RER membrane and hence the cell membrane but also provides the means for ribosome attachment to the mRNA. It is not presumed to have a direct role in fusion. Only one amino acid difference was identified between a parental Vero-adapted virus and its progeny. This was in the F protein of JL-2 (amino acid residue 230) after passage in B95a cells. The role of the mutation is unknown but it is not implicated in the smaller plaque morphology of JL-2 because the amino acid difference only occurred after passage five whereas smaller plaques were observed after the first passage. The data suggest that the F protein does not have a main role in host cell tropism.

7.5 FUTURE STUDIES

In this study it would have been useful to passage MuV in Vero cells alongside passage in the other cell lines to investigate whether the parental Vero-adapted viruses would have increased in fitness in parallel with virus passaged in B95a and HeLa cells. In the future, it would be of interest to analyse the sequence of the P, L, M and NP genes of the B95a, HeLa and MRC-5 adapted viruses to investigate whether other amino acid differences could account for the host cell adaptation. It would also be of interest to grow the variants of the Jeryl Lynn and Urabe vaccines and of Enders and Rubini in B95a and HeLa cells in competition assays to determine if one virus population would outgrow the other and to calculate the level at which variants arise, by quantitative PCR. The isolation and growth of clinically derived wild type virus in B95a cells and in HeLa cells could be an important investigation to determine if virus progeny resembles the wild type or if variants arise, as observed for isolation and passage in Vero cells, and to determine if virus progeny retains its infectivity for humans.

7.6 CONCLUSIONS

- Mumps viruses passaged in B95a or HeLa cells were of greater fitness, as determined by an increase in infectivity, measured using the $TCID_{50}$ assay, than the parental Vero-adapted viruses.
- Changes in virus phenotype were identified after passage of MuVs in B95a and HeLa cells; the HN glycoprotein is not implicated in these events.
- Neither the HN nor F glycoproteins are implicated in the apparent adaptation of JL-5 to B95a cells or of all MuVs to HeLa cells.
- Amino acids 106 and 266 within the HN protein possibly affect the neuraminidase activity of MuVs and were predicted to be exposed on the surface of the HN protein.

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APPENDIX I

THE STANDARD GENETIC CODE

UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
UUA	Leu	UCA	Ser	UAA	stop	UGA	stop
UUG	Leu	UCG	Ser	UAG	stop	UGG	Trp
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly

SYMBOLS FOR AMINO ACIDS:

SYMBOL	ABBREVIATION	FULL NAME
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

APPENDIX II

The nucleotide sequence determined of the HN gene of seven mumps viruses used in this project.

```
            1                                                    50
JL-2        ---------- ---------- ---------- ---------- -------A--
JL-5        ---------- ---------- ------C--- -----T---- ---T------
Ur PT1      ---------- T--------- ----G----- --------T- ----------
Ur PT3      ---------- T--------- ----G----- --------T- ----------
Enders      ---------- ---------- ---G------ ---------- ----------
Rubini      ---------- ---------- ---G------ ---------- ----------
Po15/t      ---------- ---------- ---------- --------T- ----------
Consensus   AAAGAAAAAA GCAAGCCAGA ACAAACTTAG GATCACAACA CAACACAGAA

            51                                                   100
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      CCCC------ C----T---- --TC--T--- -G--CGA--G ----------
Ur PT3      CCCC------ C----T---- --TC--T--- -G--CGA--G ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      CCCC------ --C------- ---C------ ---------G ----------
Consensus   TATTAGCTGC TATCACAACT GTGTTCCGGC CACTAAGAAA ATGGAGCCCT

            101                                                  150
JL-2        ---------- ---------- -----C---- ---------- ----------
JL-5        ---------- T--------- ---------- ---------- ----------
Ur PT1      -A-----C-- --C------A ---------- ---------- -------T--
Ur PT3      -A-----C-- --C------A ---------- ---------- -------T--
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      -----T-C-- --C---A--- ---------- ---------- ---G----C-
Consensus   CGAAACTATT CATAATGTCG GACAATGCCA CCTTTGCACC TGGACCTGTT

            151                                                  200
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      A-C------- -A-AC----- ---G------ -----C---- ----------
Ur PT3      A-C------- -A-AC----- ---G------ -----C---- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      A-C------- ---AC----- ---------- -----C---- ----------
Consensus   GTTAATGCGG CTGGTAAGAA GACATTCCGA ACCTGTTTCC GAATATTGGT

            201                                                  250
JL-2        ---------- -----A---- ---------- ---------- ----------
JL-5        ---------- -----A---- T--------- ---------- ----------
Ur PT1      A--G------ ---------- ---------- A--------- ----------
Ur PT3      A--G------ ---------- ---------- A--------- ----------
Enders      ---------- -----C---- ---------- ---------- ----------
Rubini      ---------- -----C---- ---------- ---------- ----------
Po15/t      A--------G ---------- ---------- ---------- ----------
Consensus   CCTATCTGTA CAAGCTGTTA CCCTTATATT GGTTATTGTC ACTTTAGGTG

            251                                                  300
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      -----G-G-- ---------- ---------- ---------- ----------
Ur PT3      -----G-G-- ---------- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      -----G-G-- ---------- ---------- ---------- ----------
Consensus   AGCTTATTAG GATGATCAAT GATCAAGGCT TGAGCAATCA GTTGTCTTCA
```

APPENDIX II continued:

```
            301                                                        350
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- G--------- ----------
Ur PT1      ---G------ ---------- G------A-- ---------- ----------
Ur PT3      ---G------ ---------- G------A-- ---------- ----------
Enders      ---------- ---------- ---------- G--------- ----------
Rubini      ---------- ---------- ---------- G--------- ---A------
Po15/t      ---------- -A--C----- G--------- ---------- ----------
Consensus   ATTACAGACA AGATAAGAGA ATCAGCTGCT ATGATTGCAT CTGCTGTGGG

            351                                                        400
JL-2        ---------- -----A---- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- --T-------
Ur PT1      ---------- ---------- -C-------- ---------- ----------
Ur PT3      ----G----- ---------- -C-------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- -C-------- ---------- ----------
Consensus   AGTAATGAAT CAAGTTATTC ATGGAGTAAC GGTATCCTTA CCCCTACAAA

            401                                                        450
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        -------T-- ---------- ---------- -------T-- ----------
Ur PT1      ---------- ---------- --G------- ---------- ------T---
Ur PT3      ---------- ---------- --G------- ---------- ------T---
Enders      -----A---- ---------- ---------- ---------- ----------
Rubini      -----A---- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- --G------- ----C----- ----------
Consensus   TTGAGGGAAA CCAAAATCAA TTATTATCCA CACTTGCCAC AATCTGCACA

            451                                                        500
JL-2        ---------- ----T----- ---------- ---------- ----------
JL-5        --------T- ---------- ------C--- ---------- --A-------
Ur PT1      GG--A---A- ---------- ---------- ---------- ----------
Ur PT3      GG--A---A- ---------- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- -C-------- ---------- ----------
Po15/t      -G--A---A- ---------- ---------- ---------- ----------
Consensus   AACAGAAACC AAGTCTCAAA CTGCTCTACA AACATCCCCT TAGTTAATGA

            501                                                        550
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- -------A-- ---------- ----------
Ur PT1      ---------- --------G- -------A-- ---------- ----------
Ur PT3      ---------- --------G- -------A-- ---------- ----------
Enders      ---------- ---------- ---------- T--------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- --------G- ---------- ---------- ----------
Consensus   CCTTAGGTTT ATAAATGGAA TCAATAAGTT CATCATTGAA GATTATGCAA

            551                                                        600
JL-2        ---------- ---C------ ---------- ---------- ----------
JL-5        ---------- ---C------ --------T- ---------- ---------C
Ur PT1      -T-------- ---------- ---------- ---------- ----------
Ur PT3      -T-------- ---------- ---------- ---------- ----------
Enders      ---------- ---C------ ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      -T-------- ---------- ---------- ---------- ----------
Consensus   CCCATGATTT CTCCTATCGGC CATCCACTCA ACATGCCTAG CTTTATCCCA
```

APPENDIX II continued:

```
            601                                                        650
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ------G--- ---------- ----------
Ur PT1      --------T- ---------- ---------- ---------- -----C----
Ur PT3      --------T- ---------- ---------- ---------- -----C----
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      --------T- ---------- ---------- ---------- -----C----
Consensus   ACTGCAACCT CACCCAATGG TTGCACAAGA ATTCCATCCT TTTCTTTAGG

            651                                                        700
JL-2        ---------T ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- -----C---- ---------- ---------- ----------
Ur PT3      ---------- -----C---- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -----C---- ---------- G--------- ----------
Consensus   TAAGACACAC TGGTGTTACA CACATAATGT AATTAATGCC AACTGCAAGG

            701                                                        750
JL-2        ---GC----- ---------- ---------- ---------- T---------
JL-5        ---------- ---C------ ---------- ---------- T-C-------
Ur PT1      ---------- G--T------ ---A------ -------A-- ------G---
Ur PT3      ---------- G--T------ ---A------ -------A-- ------G---
Enders      ---------- ---C------ ---------- ---------- ----------
Rubini      ---------- ---C------ ---------- ---------- ----------
Po15/t      ---------- G--C------ ---------- ---------- ------G--T
Consensus   ATCATACTTC ATCGAACCAA TATGTTTCCA TGGGGATTCT CGTTCAAACC

            751                                                        800
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ----T----- -------T-- ---------- ----------
Ur PT3      ---------- ----T----- -------T-- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ----T----- ---------- ---------- ----------
Consensus   GCGTCAGGGT ATCCCATGTT CAAAACCCTA AAAATCCAAT ATCTCAGTGA

            801                                                        850
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ------T--- ---------- ---------- ---------- -----A----
Ur PT3      ------T--- ---------- ---------- ---------- -----A----
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------T ---------- -----A----
Consensus   TGGCCTGAAT CGGAAAAGCT GCTCAATTGC AACAGTCCCT GATGGTTGCG

            851                                                        900
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        -G-------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ------C--- ---------- ---------- ----------
Ur PT3      ---------- ------C--- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ------C--- ----------
Rubini      ---------- ---------- ---------- ------C--- ----------
Po15/t      ---------- ---------- C--------- -C-----A-- T-----T---
Consensus   CAATGTACTG TTACGTTTCA ACTCAACTTG AAACCGACGA CTATGCGGGG
```

APPENDIX II continued:

```
            901                                                        950
JL-2        ---------- ---------- ---------- --G------- ----------
JL-5        ---------- ---------- -----T---- ---------- ----------
Ur PT1      ---------- ---------- ---------- ---------- ----T---G-
Ur PT3      ---------- ---------- ---------- ---------- ----T---G-
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- C----T---- --------G-
Consensus   TCCAGCCCAC CTACCCAGAA ACTTACCCTG TTATTCTATA ATGACACCAT

            951                                                       1000
JL-2        --A------- ---------- -G-------- ------A--- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- --A------- ---------- ----------
Ur PT3      ---------- ---------- --A------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ------GC-- ---------- ---------- G--------- ----------
Consensus   CACAGAAAGG ACAATATCTC CATCTGGTCT TGAAGGGAAT TGGGCTACTT

            1001                                                      1050
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ----T----- ---------- ---------- ----G----- A-----T---
Ur PT3      ----T----- ---------- ---------- ----G----- A-----T---
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---A------ --T------- ----G----- A---------
Consensus   TGGTGCCAGG AGTGGGGAGT GGAATATATT TCGAAAATAA GTTGATCTTT

            1051                                                      1100
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        --------C- -------A-- ---------- ---------- ------T---
Ur PT1      ---------- ---------- ---------- -----C---- ----------
Ur PT3      ---------- ---------- ---------- -----C---- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- -----C---- ------T---
Consensus   CCTGCATATG GGGGTGTCTT GCCCAATAGT ACACTAGGAG TTAAATCAGC

            1101                                                      1150
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- --------T- ---------- ---------- ----------
Ur PT3      ---------- --------T- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -----A--T- ---------- ---------C -T--------
Consensus   AAGAGAATTT TTCCGGCCCG TTAATCCATA TAATCCATGT TCAGGACCAC

            1151                                                      1200
JL-2        C--------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      -------T-- ---------- ---------- ----C----- ----------
Ur PT3      -------T-- ---------- ---------- ----C----- ----------
Enders      C--------- ---------- ---------- ---------- ----------
Rubini      C--------- ---------- ---------- ---------- ----------
Po15/t      -------T-- ---------- ---------- ----C----- ----------
Consensus   AACAAGAGTT AGATCAGCGT GCTTTGAGAT CATATTTCCC AAGTTACTTC
```

APPENDIX II continued:

```
            1201                                                    1250
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------C ---------- ---------- --------T- ----------
Ur PT1      ----A----- -A-------- ---------T ---------- ----------
Ur PT3      ----A----- -A-------- ---------T ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ----A----- -A-----A-- ---------T ---------- ---G------
Consensus   TCTAGTCGAA GGGTACAGAG TGCATTTCTG GTCTGTGCCT GGAATCAGAT

            1251                                                    1300
JL-2        ---------- ---------- ---------- ---------- --------T-
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- ---------- ---------- ----------
Ur PT3      ---------- ---------- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
Consensus   CCTAGTTACA AATTGCGAGC TAGTTGTCCC CTCAAACAAT CAGACACTGA

            1301                                                    1350
JL-2        ---------- ---------- ---------- -------G-- ----------
JL-5        ---------- ---------- ---------- -C-------- ----------
Ur PT1      ---------- ---------- ---------- ---------- ----------
Ur PT3      ---------- ---------- ---------- ---------- ----------
Enders      ---------- ---------- ---------- -------G-- ----------
Rubini      ---------- ---------- ---------- -------G-- ----------
Po15/t      ---------- -------A-- ---------- ---------- ----------
Consensus   TGGGTGCAGA AGGAAGAGTT TTATTGATCA ATAATCGACT ATTATATTAT

            1351                                                    1400
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      -----A---- -C-------- ---------- ---------- ----------
Ur PT3      -----A---- -C-------- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      -----A---- -C--T----- ---------- ---------- ----------
Consensus   CAGAGGAGTA CTAGCTGGTG GCCGTATGAA CTCCTCTATG AGATATCATT

            1401                                                    1450
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ----AC---- ---------- ---------- ----------
Ur PT3      ---------- ----AC---- ---------- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
Consensus   CACATTTACA AACTCTGGTC AATCATCTGT GAATATGTCC TGGATACCTA

            1451                                                    1500
JL-2        ---------- ---C------ ----T----- -----C---- -------A--
JL-5        ---------- ---------- ---------C ---------- ----------
Ur PT1      ---------- ---------- -----A---- ---------- ---------G
Ur PT3      ---------- ---------- -----A---- ---------- ---------G
Enders      ---------- ---------- ---------- ---------- -A--------
Rubini      ---------- ---------- ---------- ---------- -A--------
Po15/t      ---------- ---------- -----A---- -T-------- ---------G
Consensus   TATATTCATT CACTCGTCCT GGTTCGGGCA ACTGCAGTGG TGAAAATGTA
```

APPENDIX II continued:

```
            1501                                                    1550
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        -------T-- ---------- ---------- ---------- ----------
Ur PT1      --------T- CT-----G-- ---G------ ---------- ----------
Ur PT3      --------T- CT-----G-- ---G------ ---------- ----------
Enders      ---------- --------A- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      --------T- CT-----G-- ---G------ ---------- ----------
Consensus   TGCCCAACAG TCTGTGTATC AGGAGTTTAT CTTGATCCCT GGCCATTAAC

            1551                                                    1600
JL-2        ---------- --T------- ---------- ---------- ----------
JL-5        ---------A ---------- ---------- ---------- ----------
Ur PT1      ------T--- ---------- --------C- ---------- --------C-
Ur PT3      ------T--- ---------- --------C- ---------- --------C-
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------T ---------- ----------
Po15/t      ------T--- ---------- ---------- ---------- ----------
Consensus   TCCATACAGC CACCAATCAG GCATTAACAG AAATTTCTAT TTCACAGGTG

            1601                                                    1650
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- -------A-- ----------
Ur PT1      ----AT---- ---------- --T--A--A- ---------- ----------
Ur PT3      ----AT---- ---------- --T--A--A- ---------- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ----AT---- ---------- -----A--A- ---------- ----------
Consensus   CACTGCTAAA TTCAAGCACA ACCAGGGTGA ATCCTACCCT TTATGTCTCT

            1651                                                    1700
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- ---------- ---------- -------A-- -G---C----
Ur PT3      ---------- ---------- ---------- -------A-- -G---C----
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -C-------- G--------C ---------- -----C-A--
Consensus   GCCCTTAATA ATCTTAAAGT ACTAGCCCCA TATGGTACTC AAGGATTGTT

            1701                                                    1750
JL-2        ---G------ ---------- ---------- ---------- ----------
JL-5        ---T------ ---------- ---------- ---------C -----C----
Ur PT1      ------G--- ---------- ---------- ---------- --T-------
Ur PT3      ------G--- ---------- ---------- ---------- --T-------
Enders      ---A------ ---------- ---------- ---------- ----------
Rubini      ---A------ ---------- ---------- ---------- ----------
Po15/t      ------G--- ---------- ---------- ---------- --T--C----
Consensus   TGCCTCATAC ACCACAACCA CCTGCTTTCA AGATACCGGT GACGCTAGTG

            1751                                                    1800
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- -----G---- ---------- ---G------
Ur PT1      ---------- T--------- ---------- -------C-- ----------
Ur PT3      ---------- T--------- ---------- -------C-- ----------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- T--------- ---T------ -------C-- ----------
Consensus   TGTATTGTGT CTATATTATG GAACTAGCAT CGAATATTGT TGGAGAATTC
```

APPENDIX II continued:

```
            1801                                                    1850
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
Ur PT1      ---------- --------A- ---------- ---------- -CA-------
Ur PT3      ---------- --------A- ---------- ---------- -CA-------
Enders      ---------- ---------- ---------- ---------- ----------
Rubini      ---------- ---------- ---------- ---------- ----------
Po15/t      -----C---- --------A- ---------T ---------- -CA---CA--
Consensus   CAAATTCTAC CTGTGCTAGC CAGATTGACC ATCACTTGAG TTGTAGTGAA

            1851                                                    1899
JL-2        -----T---- ---------- ---------- ---------- ---------
JL-5        ---------- -------C-- ---------- --------T- ---------
Ur PT1      --C--TG--- G--CC----- -----CT--- A-CT------ ---------
Ur PT3      --C--TG--- G--CC----- -----CT--- A-CT------ ---------
Enders      ---------- ---------- ---------- ---------- ---------
Rubini      ---------- ---------- ---------- ---------- ---------
Po15/t      --C---G--G G--CC--C-- --T------- A--T------ ---------
Consensus   TGTAGCAGGA AGCTTTATGG GCGTGTCTCA TTTCTTATCG ATTATTAAG
```

The nucleotide sequence of the F gene of the MuVs. The start codon at position 64-66 is underlined, as is the stop codon at position 1678-1680. The nucleotide sequence includes a consensus sequence and the sequences of the SBL-1 (Elango *et al.*, 1989), RW (Waxham et al., 1995) and UrAm9 (Cusi *et al.*, 1995) strains of MuV.

```
            1                                                        50
JL-2        ---------- ---------- ---------C ---------- ----------
JL-5        ---------- ---------- A-----G--- ---------- ----------
UrPT1       ---------- ---C---T-- ------G--- ---------- ----------
UrPT3       ---------- ---C---T-- ------G--- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- A--------- ---------- ----------
Po15/t      ---------- ----C--T-- ------G--C ----C----- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ----C-T--- -----G---- ---C------ ----------
UrAM9                      ----T- ------G--- -C-------C ----------
Consensus   AAGCCTAGAA GGATATCCTA CTTCTCAACT TTCCAACTTT GAAAATAGAA

            51                                                       100
JL-2        ---------- ---------- ---A------ ---------- ----------
JL-5        ---------- --------C- ---------- ---------- ----A-----
UrPT1       -T-------- ---------- -----TT--- --C------A A--------G
UrPT3       -T-------- ---------- -----TT--- --C------A A--------G
ENDERS      -------C-- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      -T-------- ---------- -----TC--- --C------- A--------G
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          -T-------- ---------- -C---T---- --C------- ---------G
UrAm9       -T-------- ---------- -----TT--- --C------A A--------G
Consensus   TAGATCAGTA ATCATGAAGG CTTTTCCAGT TATTTGCTTG GGCTTTGCAA

            101                                                      150
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---C------ ----------
UrPT1       ---------- T---G----- ---------- -C-------- ----------
UrPT3       ---------- T---G----- ---------- -C-------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      -------G-- T--------- ---------- -C-------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          -------G-- T--------- ---------- -C-------- ----------
UrAm9       ---------- T---G----- ---------- ---------- ----------
Consensus   TCTTTTCATC CTCTATATGT GTGAATATCA ATATCTTGCA GCAAATTGGA

            151                                                      200
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       --T------- ----A----- ---------- ---------- ----------
UrPT3       --T------- ----A----- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      --T------- ----A----- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          --T------- ----A----- ---------- ---------- -------T-
UrAm9       --T------- ----A----- ---------- ---------- ----------
Consensus   TACATCAAGC AACAGGTCAG GCAACTAAGC TATTACTCAC AAAGTTCAAG
```

Appendix III continued:

```
            201                                                        250
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- --A------- ---------- ---------- ----------
UrPT1       -------A-- ---------- ---------- ---------- -T--------
UrPT3       -------A-- ---------- ---------- ---------- -T--------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      -------A-- ---------- ---------- ---------- ------G---
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          -------A-- ---------- ---------- ---------- ----------
UrAm9       -------A-- ---------- ---------- ---------- -T--------
Consensus   CTCCTACGTA GTGGTCAAGC TTTTACCGAA TATCCAACCC ACTGATAACA

            251                                                        300
JL-2        ---------- ---------- -T-------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ------C---
UrAm9       ---------C ---------- ---------- ---------- ----------
Consensus   GCTGTGAATT TAAGAGTGTA ACTCAATACA ATAAGACCTT GAGTAATTTG

            301                                                        350
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ------T---
UrPT1       ---------- ---------- ---------- ---------- -----T----
UrPT3       ---------- ---------- ---------- ---------- -----T----
ENDERS      ---------- ---------- ---------- ---A------ ----------
RUBINI      ---------- ---------- ---------- ---A------ ----------
Po15/t      ---------- -------G-- ---------- ---------- -T--------
SBL-1       ---------- ---------- ---------- ---A-G---- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   CTTCTTCCAA TTGCAGAAAA CATAAACAAT ATTGCATCGC CCTCACCTGG

            351                                                        400
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------G ------A--- ------T--- ---T------ ----------
UrPT3       ---------G ------A--- ------T--- ---T------ ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ------A--- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ------A--- ---------- ---------- ----------
UrAm9       ---------G ------A--- -------T-  ---T------ --------C-
Consensus   GTCAAGACGT CATAAACGGT TTGCTGGCAT TGCCATTGGC ATTGCTGCGC
```

Appendix III continued:

```
            401                                                   450
JL-2        ---------- ------G--- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- -----A---- ---------- ----------
UrPT3       ---------- ---------- -----A---- ---------- ----------
ENDERS      ---------- ------G--- ---------- ---------- ----------
RUBINI      ---------- ------G--- ---------- ---------- ----------
Po15/t      ---------- ---------- -----A---- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- --G------- ---------- ----------
UrAm9       ---------- ---------- -----A---- ---------- ----------
Consensus   TCGGTGTTGC GACCGCAGCA CAAGTGACTG CCGCTGTCTC ATTAGTTCAA

            451                                                   500
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ------G--- ---------- -------A--
UrPT3       ---------- ---------- ------G--- ---------- -------A--
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ----C----- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ------G--- ---------- ----------
UrAm9       ---------- ---------- ------G--- ---------- -------A--
Consensus   GCACAGACAA ATGCACGTGC AATAGCAGCG ATGAAAAATT CAATACAGGC

            501                                                   550
JL-2        ---------- ---------- ---------- ---------G ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------A ---------- ---------- ------T--- ----------
UrPT3       ---------A ---------- ---------- ------T--- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---C------ ---------- ---------- ---T------ ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---A------ ---------- ---------- ----------
UrAm9       ---------A ---------- ---------- ------T--- ----------
Consensus   AACTAATCGG GCAGTCTTCG AAGTGAAGGA AGGCACCCAA CAGTTAGCTA

            551                                                   600
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- --G-------
UrPT1       ---------- ---------- -----C---- ---------- ----------
UrPT3       ---------- ---------- -----C---- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- -----C---- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- -----C---- ---------- ----------
UrAM9       ---------- ---------- -----C---- ---------- ----------
Consensus   TAGCGGTACA AGCAATACAA GACCATATCA ATACTATTAT GAACACCCAA
```

Appendix III continued:

```
            601                                                   650
JL-2        ---------- ---------- ---------- ---------- -------T--
JL-5        ---------- ---------- ---------- ----A----- ----------
UrPT1       ---------- ---------- ---------- ---------- ----T-T---
UrPT3       ---------- ---------- ---------- ---------- ----T-T---
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----T-----
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ------A---
UrAm9       ---------- ---------- ---------- ---------- ----T-----
Consensus   TTGAACAATA TGTCTTGTCA GATCCTTGAT AACCAGCTTG CAACCTCCCT

            651                                                   700
JL-2        ---------- ---------- ---------- ---C------ ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- -------C--
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   AGGATTATAC CTAACAGAAT TAACAACAGT GTTTCAGCCA CAATTAATTA

            701                                                   750
JL-2        ---------- ---------- -----C---- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ----G----- ---------- ---------- ---------- ----------
UrPT3       ----G----- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ----G----- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   ATCCAGCATT GTCACCGATT AGTATACAAG CCTTGAGGTC TTTGCTTGGA

            751                                                   800
JL-2        --------A- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- C--------- ---------- -----C--A-
UrPT3       ---------- ---------- C--------- ---------- -----C--A-
ENDERS      --------A- ---------- ---------- --------C- ----------
RUBINI      --------A- ---------- ---------- --------C- ----------
Po15/t      ---------- ---------- ---------- -----C--G- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- --------G- ----------
UrAm9       ---------- ---------- C--------- ---------- ----C---A-
Consensus   AGTATGACGC CTGCAGTGGT TCAAGCAACA TTATCTACTT CAATTTCTGC
```

Appendix III continued:

```
            801                                                       850
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ------G--- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ----------T ----------
UrPT3       ---------- ---------- ---------- ---------T ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------T A---------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------T ----------
UrAm9       ---------- ---------- ---------- ---------T ----------
Consensus   TGCTGAAATA CTAAGTGCCG GTCTAATGGA GGGTCAGATA GTTTCTGTTC

            851                                                       900
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ----T----- ---T------
UrPT3       ---------- ---------- ---------- ----T----- ---T------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- -----C---- ----T----- ---T------
SBL-1       ------     ----- ---------- -----C---- ----------
RW          ---------- ---------- -----C---- ----T----- ----------
UrAm9       ---------- ---------- ---------- ----T----- ---T--C---
Consensus   TGCTAGATGA GATGCAGATG ATAGTTAAGA TAAACATTCC AACCATTGTC

            901                                                       950
JL-2        ---------- ---------- ---------- ---------- -A--T--C--
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----T-----
RUBINI      ---------- ---------- ---------- ---------- ----T-----
Po15/t      ---------- ---------- ---------- --------A- ----------
SBL-1       ---------- ---------- ---------- ---------- ----T-----
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ---------- ---------- ----------
Consensus   ACACAATCAA ATGCATTGGT GATTGACTTC TACTCAATTT CGAGCTTTAT

            951                                                      1000
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------G ---------- -C-------- ------A--- ----------
UrPT3       ---------G ---------- -C-------- ------A--- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ---------- --A-------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- C-A-------
UrAm9       ---------G ---------- ---------- ------A--- ----------
Consensus   TAATAATCAA GAATCCATAA TTCAATTGCC AGACAGGATC TTGGAGATCG
```

Appendix III continued:

```
            1001                                                    1050
JL-2        ---------- ---------- ---------- ---------C ---------T
JL-5        ----C----- ---------- ---------- ---------- ----------
UrPT1       ---------- ----A----- --------A- ---------- ----------
UrPT3       ---------- ----A----- --------A- ---------- ----------
ENDERS      -A-------- ---------- ---------- ---------- ----------
RUBINI      -A-------- ---------- ---------- ---------- ----------
Po15/t      ---------- ----A----- -----A--A- ---------- ----------
SBL-1       -A-------- ---------- ---------- ---------- ----------
RW          ---------- ----A----- --------A- ---------- ----------
UrAm9       ---------- ----A----- --------A- ---------- ----------
Consensus   GGAATGAACA ATGGCGCTAT CCAGCTAAGA ATTGTAAGTT GACAAGACAC

            1051                                                    1100
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        -----G---- ---------- ---------- ---------- ----------
UrPT1       -----C---- ---------- ---------- ---------- -----T----
UrPT3       ---------- ---------- ---------- ---------- -----T----
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----G-----
Po15/t      ---------- ---------- --- -------- --------T -----T----
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- -----T----
UrAm9       ---------- ---------- ---------- ---------T -----T----
Consensus   CACATATTCT GCCAATACAA TGAGGCAGAG AGGCTGAGCC TAGAAACAAA

            1101                                                    1150
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- -A-------- ---------- --C-------
UrPT3       ---------- ---------- -A-------- ---------- --C-------
ENDERS      ---------- ---------- ---------- ---------- T---------
RUBINI      ---------- ---------- ---------- ---------- T---------
Po15/t      ---------- ---------- -A-------- ------T--- --C-------
SBL-1       ---------- ---------- ---------- ---------- T---------
RW          ---------- -----T---- -A-------- ---------- --C-------
UrAm9       ---------- ---------- -A-------- ---------- --C-------
Consensus   ACTATGCCTT GCAGGCAATA TTAGTGCCTG TGTGTTCTCA CCTATAGCAG

            1151                                                    1200
JL-2        ----C----- ---------- -----G---- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ACG------- ---------- ----------
UrPT3       ---------- ---------- ACG------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- --------A- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------- ---------- ACG------- ---------- ----------
Consensus   GGAGTTATAT GAGGCGATTT GTAGCACTGG ATGGAACAAT TGTTGCAAAC
```

Appendix III continued:

```
            1201                                                    1250
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        --C--G---- ----A----- ---T------ ---------- ----------
UrPT1       ---------- ---------- --------A- ---------- ----------
UrPT3       ---------- ---------- --------A- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---A------ ----A----- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ----------
UrAm9       ---------C ---------- --------A- ---------- ----------
Consensus   TGTCGAAGTG TAACGTGTCT ATGCAAGAGT CCATCTTATC CTATATACCA

            1251                                                    1300
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ------A--- ----------
UrPT1       ---------- ---------- ---------- ---------- ----------
UrPT3       ---------- ---------- ---------- ------CG-- ----------
ENDERS      ---------- ---------- ---------- ------CG-- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- ------CG-- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ------CA-- ----------
UrAm9       ---------- ---------- ---------- ------CG-- ----------
Consensus   ACCTGACCAT CATGCAGTCA CGACCATTGA TCTAACGTCA TGTCAAACAT

            1301                                                    1350
JL-2        ---------- ---------- ---------- ----A----- ----------
JL-5        ---------- ---------- ---------- ---------- ---T------
UrPT1       -------A-- ----T----- ---------- ----T----- ----------
UrPT3       -------A-- ----T----- ---------- ----T----- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ---T------
Po15/t      -------A-- ---------- ---------- ----T----- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          -------A-- ----T----C ---------- ----T----- ----------
UrAm9       -------A-- ----T----- ---------- ----T----- ----------
Consensus   TGTCCCTGGA CGGACTGGAT TTCAGCATTG TCTCGCTAAG CAACATCACT

            1351                                                    1400
JL-2        ---------- ---------- ---------- -----G---- ----------
JL-5        ---------- ---------- ---------- ---------- ----C-----
UrPT1       ---------- -C-----C-- ---------- ---------- ----------
UrPT3       ---------- -C-----C-- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- C---------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- -C-----C-- ---------- ---------- -C--------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- -C-----C-- ---------- --A------- ----------
UrAm9       ---------- -C-----C-- ---------- ---------- ----------
Consensus   TACGCTGAGA ATCTTACTAT TTCATTGTCT CAGACAATCA ATACTCAACC
```

Appendix III continued:

```
            1401                                                    1450
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- --T-------
UrPT1       ------C--- --------A- ---------- ---------- ----------
UrPT3       ------C--- --------A- ---------- ---------- ----------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ------C--- --------A- ---------- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ------C--- --------A- -------A-- ---------- ----------
UrAm9       ------C--- --------A- ---------- ---------- ----------
Consensus   CATTGATATA TCAACTGAGC TGAGTAAGGT TAATGCATCC CTCCAAAATG

            1451                                                    1500
JL-2        --------- ---------- --T------- ---------- C---------
JL-5        ---------- ---------A ---------- ---------- CT-------G
UrPT1       -------G-- ------G--- ---------- -G-------- ---G------
UrPT3       -------G-- ------G--- ---------- -G-------- ---G------
ENDERS      --------- ---------- --T------- ---------- C---------
RUBINI      ---------- ---------- --T------- ---------- C---------
Po15/t      -------G-- T-----G--- ---------- ---------- ---G-----G
SBL-1       ---------- ---------- --T------- ---------- ----------
RW          -------G-- ------G--- ---------- ---------- ---G------
UrAm9       -------G-- ------G--- ---------- -G-------- ---G------
Consensus   CCGTTAAATA CATAAAAGAG AGCAACCATC AACTCCAATC TGTTAGTGTA

            1501                                                    1550
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        G--------- ---------- -------T-- -----G---- ----------
UrPT1       -AC--C---- ---------- ---------- ---------- -------T--
UrPT3       -AC--C---- ---------- ---------- ---------- -------T--
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      -A---C---- -T-------- -------T-- ---------- ----------
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          -A---C---- -T-------- ---------- ---------- ----------
UrAm9       -AC--C---- ---------- ---------- ---------- -------T--
Consensus   AGTTCTAAAA TCGGAGCTAT AATTGTAGCA GCCTTAGTTT TGAGCATCCT

            1551                                                    1600
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---A------ ---------- ---------- ---------- -----A----
UrPT3       ---A------ ---------- ---------- ---------- -----A----
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---A------ C-----A--- ---------- ---------- -----A--C-
SBL-1       ---------- ---------- ---------T ---------- ----------
RW          ---A------ ---------- ---------- ---------- -----A----
UrAm9       ---A------ ---------- ---------- ---------- -----A----
Consensus   GTCGATTATC ATTTCGCTAT TGTTTTGCTG CTGGGCTTAC ATTGCGACTA
```

Appendix III continued:

```
            1601                                                    1650
JL-2        ---------- ---------- ---------- ---------- ----------
JL-5        ---------- ---------- ---------- ---------- ----------
UrPT1       ---------- ---------- ---------- ---------- T---------
UrPT3       ---------- ---------- ---------- ---------- T---------
ENDERS      ---------- ---------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      -------T-- ---------- ---------- ---------- ----T-----
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- ---------- ---------G
UrAm9       ---------- ---------- ---------- ---------- T---------
Consensus   AAGAAATCAG AAGAATCAAC TTCAAAACAA ATCATATCAA CACAATATCA

            1651                                                    1700
JL-2        ---------- ---------- ---------- ---------- -------A--
JL-5        ---------- ---------- ---------- -C---G---- ----------
UrPT1       ---------- ---------- T--------- -CC---CA-- -------A--
UrPT3       ---------- ---------- T--------- -CC---CA-- -------A--
ENDERS      ---------- G--------- ---------- ---------- ----------
RUBINI      ---------- ---------- ---------- ---------- ----------
Po15/t      ---------- ---------- ---------- -C----CA-- -------A--
SBL-1       ---------- ---------- ---------- ---------- ----------
RW          ---------- ---------- ---------- -C----CA-- ----------
UrAm9       ---------- ---------- T--------- -CC---C--T -------A--
Consensus   AGTAGTGTCG ATGATCTCAT CAGGTACTAA TTTTAAATTG GTGATTCGTC

            1701
JL-2        ---------A -----G---- -
JL-5        --------TT ---------- -
UrPT1       -----T-G-- ---------- -
UrPT3       -----T-G-- ---------- -
ENDERS      ---------A ---------- -
RUBINI      ---------A ---------- -
Po15/t      -----T-C-- ---------- -
SBL-1       --------G- ---------- -
RW          -----T-CG- ---------- -
UrAm9       -----T-G-- ---------- -
Consensus   CTGCAATTAG AAAAGATTTA G
```